COMBINATION THERAPY FOR HYPOTHYROIDISM

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COMBINATION THERAPY FOR HYPOTHYROIDISM

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EDITORIAL
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Editorial: Combination Therapy for Hypothyroidism: The Journey From Bench to Bedside

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Keywords: hypothyroidism, levothyroxine, combination therapy, patient outcomes, clinical trials, basic and translational research

Editorial on the Research Topic

Combination Therapy for Hypothyroidism

The debate about the optimum replacement therapy for hypothyroidism has been active for many years, and continues to be an area of interest for basic and clinical researchers, practicing clinicians, and patients. In this Research Topic "combination therapy for hypothyroidism," we bring together a collection of 16 diverse articles describing basic research, clinical research, hypotheses, and opinion pieces that are woven together to illustrate the rich tapestry of this topic.

The importance of thyroid hormone for optimal function of many different organ systems is well-documented. Within the articles included in this topic are three articles exploring the importance of thyroid hormone for cardiometabolic function. One article by Forini et al. discusses whether disordered epigenetic remodeling of chromatin structure and interplay with non-coding RNA may contribute to the cardiac dysfunction seen in low triiodothyronine (T3) states and discusses how the appropriately timed reversal of the low T3 state may improve cardiac function. Another article by Mastorci et al. also reviews the cardioprotective effects of T3, including pathways involving the non-genomic effects of T3. A third article by Stamatouli et al. reviews the effect of thyroid hormone deficiency on diverse cardiometabolic clinical indices such as impaired lipid profile, chronic inflammation, increased oxidative stress, and increased insulin resistance.

The importance of individualized therapy for hypothyroidism is accented in an analysis by Hoermann et al.. By analyzing the thyroid analytes in patients being treated with levothyroxine, intra-individual clustering of thyroid parameters was revealed, although such clustering tended to be masked when data from many individuals were averaged, perhaps leading to consideration of personalized treatment goals during therapy. A treatise by Köhrle et al. discusses measurement of the thyroid hormone metabolite 3,5 diiothyronine. These authors describe altered concentration of 3,5 diiothyronine with certain chronic illnesses. They speculate as to whether altered concentrations of 3,5 diiothyronine could contribute to dissatisfaction with levothyroxine monotherapy. Measurement of another thyroid hormone metabolite, reverse T3, has always been a controversial topic and has been of interest to subsets of clinicians and patients. Gomes-Lima et al. review the small amount of literature addressing this topic and conclude that there is insufficient evidence to suggest that measurement of reverse T3 might be useful for guiding combination therapy.

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An investigation reported by Jonklaas et al. based on a survey of American Thyroid Association members illustrates that not only do physicians prescribe combination therapy for their patients, but that also they are prescribing more combination therapy over time. A review by McAninch and Bianco provides some history of the use of thyroid hormone preparations. Their discussion includes the potential personalization of thyroid hormone treatment based on an individual's type 2 deiodinase genotype and the possibility that individuals homozygous for the Thr92Ala polymorphism may have impaired thyroid signaling.

Patient reported outcomes and patient preferences are important when assessing the individual patient's response to their hypothyroidism therapy, and these parameters will no doubt be key outcomes in any future combination therapy trials. One patient-reported outcome discussed in this topic collection is a novel hypothyroidism symptoms scale described and tested by Brokhin et al.. A meta-analysis by Akirov et al. shows that patient preference for combination therapy does not differ from that which would be expected by chance, thus illustrating that careful design of future combination therapy trials is essential to fully explore patient preferences.

A review provided by Taylor et al. explores dissatisfaction with levothyroxine therapy, and discusses both the current approach that could be taken to therapeutic trials of liothyronine in individual patients and also potential biomarkers that could be utilized to guide use of combination therapy. They also touch upon the factors that would be important to consider in any future trials of combination therapy. Using computer modeling, another analysis by DiStefano and Jonklaas examines the residual endogenous thyroid function of individuals being treated with thyroid hormone and concludes that the varying degrees of residual thyroid function retained by patients may be one of the variables contributing to the heterogeneous results obtained from the published studies of combination therapy.

Two complementary articles by Cappola and Madan and Celi et al. address our current equipoise with respect to trials of combination therapy and outline the attributes that the authors believe are critical to avoid the shortcomings of previously published combination therapy trials, and to ensure the success of future trials. In addition to the need for randomization, blinding, and placebo-control, both these articles stress the importance of study population, dosing strategy, appropriate selection of primary and secondary outcomes, and adequate statistical power.

Two very different approaches to attempting to develop a sustained release triiodothyronine (T3) or liothyronine (LT3) preparation are illustrated in the review by Idrees et al. and the original research article by Santini et al.. In the former the authors describe a number of products, including a poly-zinc-LT3 formulation that has been tested in rats and may be entering phase I trials in humans within a few years (Idrees et al.), while in the latter the investigators show that stable serum levels of T3 can be achieved by administration of the non-deiodinative T3 metabolite, T3-sulfate (Santini et al.).

In summary, the articles in this collection examine tissue-specific aspects thyroid hormone action, potential laboratory markers of thyroid hormone action, personalization of thyroid hormone therapy, patient-reported outcomes that include patient satisfaction, and physician practices. They also explore potential design of future combination therapy trials and the possibility of a sustained release T3 preparation that may allow fulfillment of the promise of physiologic dosing of combination therapy. We hope that these articles will help clinicians consider whether they wish to offer combination therapy to their patients, and what parameters they would follow to gauge the success of the therapy. We also hope that this collection of articles will provide a framework for researchers designing future trials of combination therapy to determine if there are indeed patients for whom combination therapy is a more satisfactory treatment.

AUTHOR CONTRIBUTIONS

JJ, AC, and FC each contributed to the conception and manuscript preparation for this editorial. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Short-Term Time Trends in Prescribing Therapy for Hypothyroidism: Results of a Survey of American Thyroid Association Members

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Objective: Hypothyroid patients frequently request specific therapies from their physicians. Combination therapy is vigorously discussed at professional meetings. We wished to determine if physician prescribing patterns for hypothyroidism changed during 2017 after specific educational events.

Methods: A survey addressing treatment of hypothyroidism was emailed to American Thyroid Association (ATA) members on three occasions in 2017. The Spring emails were sent prior to a satellite symposium addressing hypothyroidism, and prior to the annual Endocrine Society and ATA meetings; the December emails were sent after these events. Physicians were presented with thirteen theoretical patients and chose from 6 therapeutic options, including levothyroxine, synthetic combination therapy, thyroid extract, and liothyronine monotherapy. The patient scenarios successively incorporated factors potentially providing reasons for considering combination therapy. Multivariate repeated measures logistic regression analyses first examined effects of physician characteristics on prescribing the various therapies. Then, analyses also incorporated timing, by comparing prescribing patterns in February, March, and December.

Results: In analyses of prescribing levothyroxine monotherapy vs. any T3 therapy, there was a trend of borderline significance ($p = 0.053$) for T3 therapy to be prescribed more in December compared with February-March combined. When multivariate analyses were performed controlling for time and physician characteristics, choice of therapy was only significantly affected by country of practice (OR 1.7, CI 1.3–2.2). Physician choice of therapies was also examined for the options of continuing (1) levothyroxine, vs. (2) increasing levothyroxine, (3) adding liothyronine either with or without levothyroxine reduction, or (4) replacing levothyroxine with desiccated thyroid extract or liothyronine. When multivariate analyses incorporating time and physician characteristics were performed, respondents in December (OR 1.5, CI 1.0–2.3) and those practicing in North America (OR 1.8, CI 1.2–2.6) were more likely to prescribe liothyronine.

Conclusions: This survey shows that although current North American guidelines do not recommend combination therapy, such therapy is being prescribed more over time and is also more commonly prescribed in North America. It is possible our guidelines are failing

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to incorporate evidence that physicians are considering when prescribing combination therapy. Such evidence could include data about patient preferences, and this needs to be a focus of future studies.

Keywords: hypothyroidism, combination therapy, liothyronine, thyroid extract, trends over time

INTRODUCTION

Society guidelines concerning the treatment of hypothyroidism originate from both North America and Europe, and have been published during a time period spanning 2012–2016. In 2012 one guideline from Europe (1), one from North America (2) and one narrative review authored by American and European experts (3) were published. The European guidelines (1) and the narrative review (3) both suggested that therapy combining levothyroxine (LT4) and liothyronine (LT3) could be considered under certain specific circumstances, whereas the co-authored 2012 American Association of Clinical Endocrinologists (AACE)/ American Thyroid Association (ATA) guidelines (2) did not recommend combination therapy. Updated ATA Guidelines for the Treatment of Hypothyroidism were published in 2014 (4). These guidelines concluded that there was insufficient evidence to recommend combination therapy (4).

The majority of the clinical studies of synthetic combination therapy upon which the various guidelines have based their recommendations were published between 1999 and 2009 (5–17). A single trial of therapy with desiccated thyroid extract was published in 2013 (18). Since the publication of the 2014 guidelines, one additional original research study, which did not identify an advantage of combination therapy has been published (19). In addition, subsequently published British Thyroid Association guidelines (20) have suggested that combination therapy could be prescribed and carefully monitored under certain circumstances. Most recently, the Italian Endocrine Society and the Italian Association of Clinical Endocrinologists have also suggested that combination therapy could be considered (21, 22). None of these guidelines have supported the use of desiccated thyroid extract (DTE).

From a consideration of the various guidelines, it appears that rather than seeing a trend over time toward encouraging or discouraging combination therapy, there has simply been a fluctuation in time. However, it does appear that European guidelines, generally authored by physicians practicing in Europe, have favored consideration of combination therapy. At the present, other than the one additional clinical study already mentioned (19), there have not been additional randomized clinical trials of combination therapy that might potentially alter physician prescribing. However, some studies about patient preference have been added to the literature (23–25). Previous studies from our group have shown that patient and physician characteristics affect the tendency to prescribe combination therapy (26, 27). The goal of this particular analysis was to assess the effect of short term time trends in prescribing patterns. This report compares the prescribing pattern of physicians surveyed in early 2017 and then again in late 2017. Intervening in between these two deployments of the survey was an ATA satellite symposium dedicated to the treatment of hypothyroidism that was offered prior to the 2017 annual Endocrine Society meeting, the Endocrine Society meeting itself, and the 2017 annual meeting of the ATA.

METHODS

Survey Content and Distribution

This survey of ATA members was designed to determine their choice of therapy for hypothyroidism when presented with several different theoretical patients. The study was approved by the Georgetown University Institutional Review Board and the survey questions are included as **Supplemental Material**. A link for the survey was distributed to all ATA members via email on several occasions in 2017. The survey link was distributed in February 2017, March 2017, and December 2017. The introduction to the first survey deployment outlined the goals of the survey and explained that the survey would be distributed again after key professional meetings had occurred. The introduction to the March survey had the same explanation, but stressed that those who had responded in February need not take the survey again. The two December deployments of the survey reiterated its goals and specifically invited those who had responded previously to re-take the survey.

The index patient was a 29-year old female with Hashimoto's hypothyroidism who had no specific complaints while taking LT4 replacement therapy. Her vital signs were normal and her body mass index was 25 kg/m2. She was described as having overt hypothyroidism of at least 5 years duration, being compliant with therapy, and not considering pregnancy. Her biochemical assessment showed a thyroid stimulating hormone (TSH) value of 2.2 mIU/L (normal range 0.4–4.0 mIU/L), a free thyroxine (FT4) value of 1.3 ng/dL (normal range 0.8–1.8 ng/dL), and a triiodothyronine (T3) value of 120 ng/dL (normal range 80–180 ng/dL). Twelve additional patient scenarios then introduced factors that have been suggested in the literature to potentially provide reasons for considering combination therapy. Examples of these factors included presence of symptoms, low serum T3 concentration, a patient request for T3, documentation of deiodinase polymorphism status (28–30) etc (see **Table 1**), table also included in prior reports (26, 27). Survey respondents were asked to select from the following treatment options for each of the 13 patient scenarios presented: (a) Continue current levothyroxine, (b) Increase levothyroxine dose, (c) Add 2.5 mcg liothyronine (Cytomel) twice daily and reduce levothyroxine, (d) Add 2.5 mcg liothyronine (Cytomel) twice daily to current levothyroxine, (e) Replace levothyroxine with thyroid extract (e.g., armor thyroid), (f) Replace levothyroxine with liothyronine (Cytomel) as single therapy (see left hand columns of **Tables 2A**,**B**).

TABLE 1 | Patient characteristics in questions 5–17*.

(A) Patient characteristics	Characteristics present in question stem according to question number												
	Q5	**Q6**	**Q7**	**Q8**	**Q9**	**Q10**	**Q11**	**Q12**	**Q13**	**Q14**	**Q15**	**Q16**	**Q17**
Symptoms	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Serum TSH (mIU/L)	2.2	2.2	3.9	2.2	3.9	2.2	2.2	2.2	2.2	2.2	2.2	2.2	2.2
Serum T3 (ng/dL)	120	120	120	75	75	75	75	75	75	75	75	75	75
Requests LT3	No	No	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Athyreotic	No	No	No	No	No	No	Yes	No	No	No	No	No	No
LT3 preference	No	No	No	No	No	No	No	Yes	No	No	No	No	No
Male	No	No	No	No	No	No	No	No	Yes	No	No	No	No
Polymorphism	No	No	No	No	No	No	No	No	No	Yes	No	No	No
Age	29	29	29	29	29	29	29	29	29	29	59	29	59
BMI	25	25	25	25	25	25	25	25	25	25	25	32	25
Comorbidity	No	No	No	No	No	No	No	No	No	No	No	No	Yes

*Question numbers refer to those used in the survey provided in the **Supplementary Material**. Each question incorporates the patient characteristics in the left-hand column, as indicated by each column in the body of the table.*

TABLE 2A | Response to questions regarding therapy (Q5–Q10).

	Percentage of respondents choosing each treatment option					
Question #	Q5	Q6	Q7	Q8	Q9	Q10
Patient characteristics	Feels well, TSH 2.2, T3 120	Sxs, TSH 2.2, T3 120	Sxs, TSH 3.9, T3 120	Sxs, TSH 2.2, T3 75	Sxs, TSH 3.9, T3 75	Sxs, request, TSH 2.2, T3 75
Treatment Options	**Feb**					
Continue LT4	97.56	61.85	23.25	45.20	14.23	32.40
Increase LT4	1.22	18.88	69.52	17.60	64.08	10.80
Add LT3, ↓LT4	0.41	11.24	0.40	17.60	2.81	32.00
Add LT3 to LT4	0.41	6.02	5.62	16.00	17.27	17.60
Switch to DTE	0.41	1.61	1.20	3.60	1.20	7.20
LT3 only	0.00	0.40	0.00	0.00	0.40	0.00
Treatment Options	**Mar**					
Continue LT4	98.25	61.06	21.18	42.60	15.79	30.97
Increase LT4	1.75	18.58	69.79	21.16	62.88	7.96
Add LT3, ↓LT4	0.00	12.39	2.02	20.46	3.26	38.05
Add LT3 to LT4	0.00	7.08	7.02	13.16	17.19	18.58
Switch to DTE	0.00	0.88	0.00	2.63	0.88	4.42
LT3 only	0.00	0.00	0.00	0.00	0.00	0.00
Treatment Options	**Dec**					
Continue LT4	97.66	50.39	11.72	34.13	10.16	24.60
Increase LT4	2.34	25.20	72.91	23.02	59.28	11.11
Add LT3, ↓LT4	0.00	16.54	2.91	20.63	7.91	36.51
Add LT3 to LT4	0.00	6.30	9.47	19.84	21.88	24.60
Switch to DTE	0.00	1.57	0.00	1.59	0.78	3.17
LT3 only	0.00	0.00	0.00	0.79	0.00	0.00

Statistical Analysis

The goals of the survey were to determine whether (i) patient characteristics and (ii) physician characteristics affected choice of therapy for patients (26, 27), and (iii) whether these choices changed over time. The goal of this particular analysis was to determine whether these choices changed over time. The results of the survey are initially presented as the percentage of survey respondents selecting each therapeutic option for the 13 different patient scenarios. Two different treatments of the data were then applied. The first was a binary analysis examining whether a respondent would prescribe LT4 vs. any therapy other than LT4. The second examined the prescribing

TABLE 2B | Response to questions regarding therapy (Q11–Q17).

	Percentage of Respondents Choosing Each Treatment Option						
Question #	Q11	Q12	Q13	Q14	Q15	Q16	Q17
Patient characteristics	Sxs, request, thyX, TSH 2.2, T3 75	Sxs, request, prior LT3, TSH 2.2, T3 75	Sxs, request, male, TSH 2.2, T3 75	Sxs, request, polym, TSH 2.2, T3 75	Sxs, request, 59 yo, TSH 2.2, T3 75	Sxs, request, BMI 32, TSH 2.2, T3 75	Sxs, request, co-morb, TSH 2.2, T3 75
Treatment Options	**Feb**						
Continue LT4	27.71	25.40	31.73	15.60	39.36	28.80	47.20
Increase LT4	14.06	4.60	13.25	8.00	10.44	12.80	10.00
Add LT3, ↓LT4	31.33	42.80	30.92	40.80	34.14	30.40	30.00
Add LT3 to LT4	21.29	22.00	18.88	27.20	10.84	22.00	8.00
Switch to DTE	5.62	5.20	5.22	4.00	5.22	5.60	4.40
LT3 only	0.00	0.00	0.00	4.40	0.00	0.40	0.40
Treatment Options	**Mar**						
Continue LT4	28.95	27.43	30.70	18.58	38.94	31.58	46.49
Increase LT4	10.53	6.19	9.65	4.42	12.39	10.53	10.53
Add LT3, ↓LT4	33.33	38.05	38.60	43.36	31.86	34.21	28.07
Add LT3 to LT4	21.93	25.66	17.54	31.86	12.39	19.30	10.53
Switch to DTE	5.26	2.65	3.51	1.77	4.42	4.39	4.39
LT3 only	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Treatment Options	**Dec**						
Continue LT4	18.75	19.53	21.88	14.17	28.35	21.88	41.41
Increase LT4	17.19	6.25	13.28	2.36	10.24	15.63	7.81
Add LT3, ↓LT4	38.28	46.88	38.28	43.31	45.67	34.38	35.16
Add LT3 to LT4	24.22	27.34	25.00	37.80	14.17	26.56	13.28
Switch to DTE	1.56	0.00	1.56	1.57	1.57	1.56	2.34
LT3 only	0.00	0.00	0.00	0.79	0.00	0.00	0.00

choice with the therapies categorized into four groups (1–4).

For the binary analysis, repeated measures logistic regression analysis was used to examine the relationship between the treatment chosen and patient and physician characteristics, and between physician characteristics and time of the survey (February compared with March, February compared with December, and February-March compared with December). The February vs. March comparison was performed as an internal control, as no change would be expected in this time period. Choice of either continuing or increasing LT4 (options a or b) was used as the reference and compared with choice of anything other than LT4 (choices c, d, e and f from the prescription options). The method of generalized estimating equations (GEE) was used to account for correlations among the 13 responses from the same physician. Multivariate repeated measures logistic regression analysis was also conducted controlling for patient and physician characteristics, and physician characteristics and time of the survey.

For the second analysis the response options were grouped into 4 groups as follows: group 1: continue LT4 (option a), group 2: increase LT4 (option b), group 3: add 2.5 mcg liothyronine both with or without LT4 reduction (options c and d), and group 4: replace LT4 with DTE or LT3 (options e and f). The grouping of the response options was utilized due to the small numbers of these options chosen for some patient scenarios. The choice to continue current LT4 was used as the reference. Repeated measures multinomial logistic regression analysis was used to adjust for correlations among responses from the same physician, while examining the relationship between the therapy chosen and patient and physician characteristics, and between physician characteristics and time of survey [February (reference) vs. March, February vs. December, and February-March vs. December]. The February vs. March comparison was again performed as an internal control, as no change would be expected in this time period. Multivariate repeated measures multinomial logistic regression analysis was also conducted controlling for patient and physician characteristics, and between physician characteristics and time of survey.

For both analyses, odds ratios with corresponding 95% confidence intervals and *p*-values were calculated. Statistical significance was defined as $P < 0.05$. *P*-values of between 0.05 and 1.0 were considered as trendwise or of borderline significance. An in-depth analysis of the effect of patient and physician characteristics has been reported (26, 27).

RESULTS

Physician Respondents

There were 249, 114, and 128 eligible responses to the survey from physicians who routinely prescribed therapy for hypothyroidism in February, March, and December of 2017,

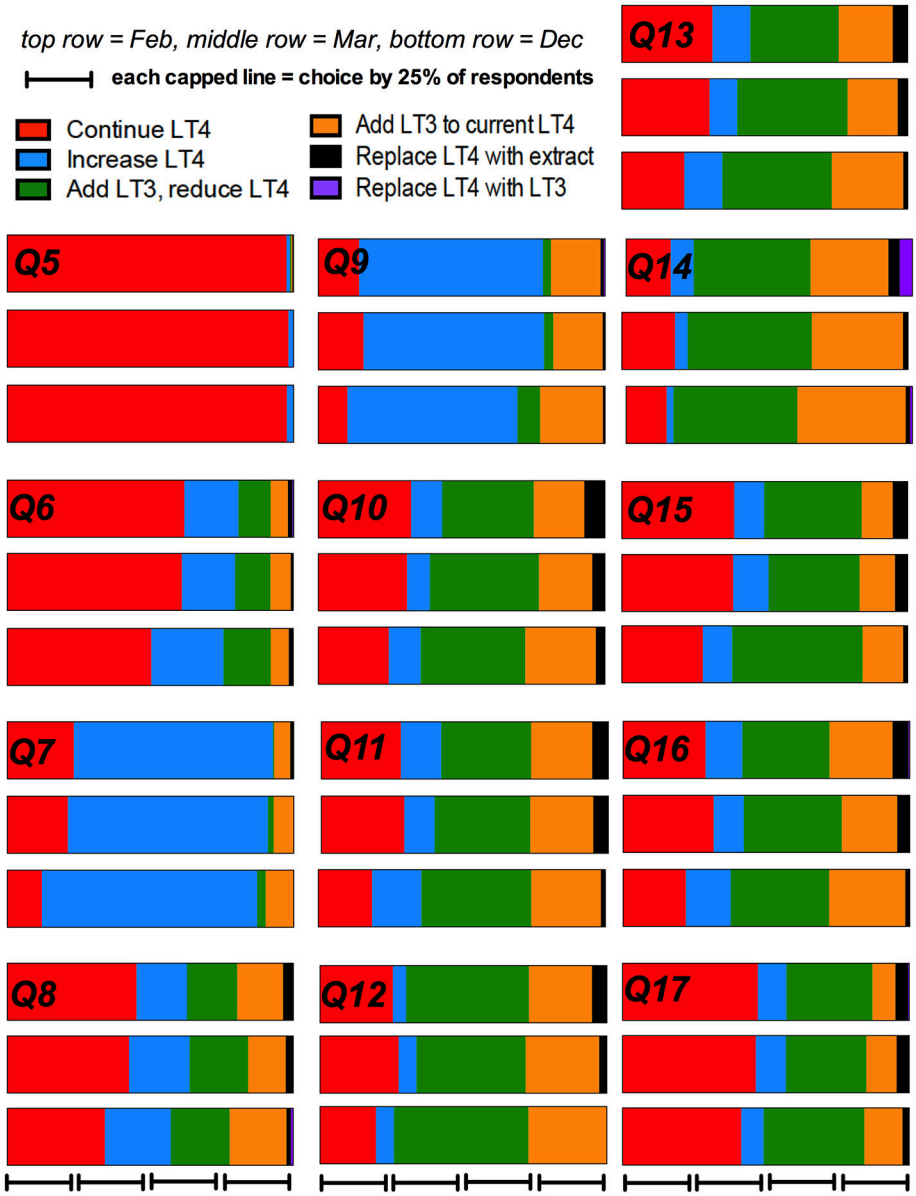

FIGURE 1 | Response to patient scenarios over time.

respectively. IP addresses were used to ensure that there were no duplicate responses in February and March, and that all December respondents had previously answered the survey in the Spring.

The responses rates from the 1,798 members of the ATA in 2017 are 14, 6.3, and 7.1%, respectively. Thus, there were 363 responses to the survey when it was initially deployed in February-March and 128 responses to the December deployment. The responding physicians were 83–88% endocrinologists, 58–75% were from North America and 12–20% were from Europe. Seventeen-twenty five percent had been in practice for 11–20 years, and 49–62% had been in practice for more than 20 years (see **Table 3**).

Descriptive Findings for the Patient Scenarios

The percentage of physician respondents choosing each individual treatment option at each of the three time points broken down by the 13 different patient scenarios is shown in **Tables 2A**,**B**. These data are also displayed graphically in **Figure 1**.

Patient and Physician Characteristics

Analysis With Binary Therapeutic Options for the 363 First Time Respondents

Multivariate repeated measures logistic regression analysis was conducted to control for all patient and physician characteristics.

TABLE 3 | Characteristics of physicians responding to survey.

Question regarding physician characteristic	Response options	% at Survey time point 1	% at Survey time point 2	% at Survey time point 3	% ATA composition in 2017
Do you prescribe and adjust LT4 therapy for patients with hypothyroidism?	Yes	100*	100*	100*	-
	No	0	0	0	-
How many years have you been in practice?	In training	2.8	2.6	1.6	-
	<5 years	9.2	7.9	7.8	-
	5–10 years	14.0	9.7	11.7	-
	11–20 years	24.8	17.5	21.1	-
	>20 years	49.20	62.3	57.8	-
Where do you practice?	North America	58.4	74.6	73.4	74
	South America	7.6	4.4	1.6	3
	Europe	20	12.3	15.6	9
	Asia	8.4	7.0	7.0	12
	Other	5.6	1.8	2.3	1
Which best describes your specialty?	Endocrinologist	87.6	82.5	83.6	63
	Surgeon	4.8	4.4	8.6	18
	Nuclear Medicine Physician	3.2	6.1	3.9	3
	Internist or Primary Care Physician	1.2	2.6	0.8	17
	Other	3.2	4.4	3.1	

** results only reported for those who answered yes.*

Patient symptoms, T3 levels, TSH levels, presence of a polymorphism, request for T3 therapy, and a stated preference for T3 therapy made it more likely that a physician would prescribe a therapy other than LT4 monotherapy (i.e., T3-containing therapy), with a $p < 0.0001$ in each case. Older age and presence of a comorbidity made it significantly more likely the physician would prescribe LT4 ($p < 0.0001$ and 0.0002, respectively). With respect to physician characteristics only country of practice affected prescribing pattern. Physicians practicing in North America were more likely to prescribe therapy other than LT4, compared with physicians from other regions ($p < 0.0001$). (see Tables 4, 3b respectively) in previously published reports (26, 27).

Analysis With Multiple Therapeutic Options for the 363 First Time Respondents

When multivariate logistic regression analyses were performed to determine whether patient characteristics affected whether physicians would prescribe continued LT4 (group 1 option) vs. increasing LT4 (group 2 option) vs. adding LT3 to the same or reduced LT4 (group 3 options) vs. replacing LT4 with T3-containing therapy comprised of either DTE or LT3 (group 4 options) most patient characteristics (patient symptoms, T3 levels, TSH levels, presence of a polymorphism, request for T3 therapy, and a stated preference for T3 therapy) appeared to be significant in the model ($p < 0.0001$). Older age and presence of a comorbidity made it significantly more likely the physician would continue LT4 ($p < 0.0002$ and 0.04, respectively). When multivariate analyses of physician characteristics were performed only country of practice was significant, with physicians practicing in North America being more likely to add LT3 to LT4 (OR 1.9, CI 1.2–2.9) and more likely to prescribe DTE or LT3 monotherapy (OR 1.7, CI 1.0–2.9). (see Tables 6, 4b, respectively) as previously published reports (26, 27).

Trends Over Time

Analysis With Binary Therapeutic Options

The prescribing trends over time are shown graphically in **Figure 1**. In univariate analysis of the binary option of prescribing LT4 vs. other therapies, a comparison of the results from respondents who completed the survey in Feb and March, compared with those who completed the survey in December showed there was a non-significant trend for physicians to prescribe therapy other than LT4 (OR 1.28, 95% CI 0.99–1.65, $p = 0.053$) (see **Table 4**). Comparison of February with March and then February with December showed OR 1.01, 95% CI 0.76–1.34, $p = 0.95$, and OR 1.29, 95% CI 0.99–1.69, $p = 0.062$, respectively. Additionally, the physician country of practice appeared to have a significant effect on choice of therapy (OR 1.7, CI 1.4–2.2, $p < 0.0001$). However, when multivariate analysis was performed controlling for time (Dec vs. Feb-Mar), and physician characteristics, choice of therapy was only significantly affected by country of practice (OR 1.7, CI 1.3–2.2, $p < 0.0001$), and time no longer showed a trend.

TABLE 4 | Univariate analyses of the effect of timing on physician prescribing of LT4 vs. any T3-containing therapy.

Time	Odds ratio	95% Confidence interval		*P*-value
March (Feb = ref)	1.01	0.76	1.34	0.9531
Dec (Feb = ref)	1.29	0.99	1.69	0.0616
December (Feb-Mar = ref)	1.28	0.99	1.65	0.053

TABLE 5 | Univariate analysis of the effect of timing on physician prescribing continued LT4 vs. increasing LT4 vs. adding LT3 to LT4 vs. replacing LT4 with T3-containing therapy.

Time	Grouping of therapeutic options	Odds ratio	95% Confidence interval		*P*-value
March (Feb = ref)	1 vs. 2	1.03	0.8	1.4	0.83
	1 vs. 3	1.1	0.7	1.7	0.70
	1 vs. 4	0.98	0.6	1.6	0.94
Dec (Feb = ref)	1 vs. 2	1.3	0.98	1.8	0.054
	1 vs. 3	**1.6**	**1.1**	**2.5**	**0.023**
	1 vs. 4	1.6	0.97	2.5	0.062
December (Feb-Mar = ref)	**1 vs. 2**	**1.3**	**1.003**	**1.8**	**0.048**
	1 vs. 3	**1.6**	**1.1**	**2.3**	**0.022**
	1 vs. 4	**1.6**	**1.01**	**2.5**	**0.045**

Continuing LT4 = therapeutic group 1 (reference), Increasing LT4 = therapeutic group 2, Adding LT3 to same or reduced LT4 = group 3, Replacing LT4 with DTE or LT3 = group 4. The options in bold font have significant p-values.

Analysis With Multiple Therapeutic Options

Physician choice of therapies over time was examined for the grouped options of (1) continuing LT4 (option a), vs. either (2) increasing LT4 (option b), (3) adding 2.5 mcg liothyronine either with or without LT4 reduction (options c and d), and (4) replacing LT4 with DTE or LT3 (options e and f). In univariate analyses in which the February survey results were used as the reference and compared with the responses in March and December, physicians were more likely to add LT3 therapy when surveyed in December (OR 1.6, CI 1.1–2.5, $p = 0.023$) (see **Table 5**). In univariate analyses, choice of therapy over time also appeared to be influenced by years in practice ($p = 0.01$), country of practice ($p = 0.0077$) and specialty ($p = 0.025$). When multivariate analyses incorporating time of survey, years in practice, country of practice, and specialty were performed, respondents were more likely to prescribe LT3 in December ($p = 0.04$), those in practice for 11–20 years were more likely to increase LT4 dosage ($p = 0.025$), those practicing in North America were more likely to prescribe LT3 ($p = 0.003$) and surgeons were more likely to increase LT4 dosage ($p = 0.028$) (see **Table 6**).

DISCUSSION

Current guidelines for treatment of hypothyroidism consider LT4 to be standard of care, and the accumulated studies of combination therapy have not shown a benefit of combination therapy. Although a proportion of patients are dissatisfied with LT4, high quality studies have not yet been performed to determine whether careful, individualized LT4 dose titration may improve the symptoms of some of these patients. Current media attention to combination therapy makes it challenging to determine the relative impact of media coverage or true patient preference on patient requests for combination therapy. This analysis shows that physicians were more likely to prescribe LT3, either added to the same LT4 dose or added to a reduced LT4 dose, when surveyed in December 2017 compared with February and March 2017. Physicians practicing in North America were also more likely to prescribe such therapy compared with those practicing in other regions.

Considering the greater prescribing of LT3 in late 2017, compared with earlier in the year, other studies have reported data about prescribing patterns at various points in time. An observational study conducted in Scotland showed that 400 out of 34,355 patients (0.11%) had been prescribed LT3 during the period 1997–2014 (31). A survey about the treatment of hypothyroidism conducted in 2013 found that 0.8% of physicians would routinely use combination therapy for treating hypothyroidism, whereas 3.6% would use such therapy in a patient with persistent symptoms (32). A study conducted 3 years later in 2016 also showed that 4.2% of physicians would prescribe combination therapy for a patient with persistent symptoms consistent with hypothyroidism (33). However, although these latter two studies used the same survey instrument, it is difficult to utilize these data to determine trends as one study surveyed physicians practicing primarily in America and Europe and the respondents were primarily Endocrinologists (32), whereas the other survey queried mostly primary care physicians and was conducted in India (33). The finding that 3.6–4.2% of physicians were willing to prescribe combination therapy in both these studies contrasts markedly with the present findings that up to 47% of physicians would add LT3 therapy while reducing the LT4 dose, depending on the specific patient scenario, and that up to 38% would add LT3 therapy while maintaining the LT4 dose, again depending upon the patient characteristics. An observational study derived from pharmacy data did show that following the publication of the European Thyroid Association Guidelines (1), which stated that combination therapy could be considered under specific circumstances, there was a trend for increasing numbers of LT3 and DTE prescriptions to be received at a specific pharmacy in Denmark (34).

The results of the current survey show that approximately one third of physicians treating patients with hypothyroidism are willing in theory to prescribe therapies other than LT4. This is despite the fact that ATA guidelines for the treatment of hypothyroidism conclude that there is insufficient evidence to support prescribing T3-containing therapies (2, 4), but in keeping with more recent recommendations from British and Italian Societies (20–22). It is difficult to identify original published data that might account for the current willingness to prescribe combination therapy, and published data about preference or improvement of symptoms with such therapy is entirely from uncontrolled (23), sparsely documented (25)

TABLE 6 | Multivariate analysis of the effect of timing on physician prescribing continued LT4 vs. increasing LT4 vs. adding LT3 to LT4 vs. replacing LT4 with T3-containing therapy.

Timing and Physician Characteristics		Treatment group	Adjusted OR	95% confidence limits	
Time (Feb=ref)	Dec	2 vs. 1	1.3	0.95	1.8
	Dec	**3 vs. 1**	**1.5**	**1.0**	**2.3**
	Dec	4 vs. 1	1.4	0.87	2.3
	Mar	2 vs. 1	1.1	0.76	1.5
	Mar	3 vs. 1	1.1	0.72	1.7
	Mar	4 vs. 1	0.92	0.55	1.5
Number of years in practice (in training = ref)	<5 years	2 vs. 1	1.7	0.65	4.3
	<5 years	3 vs. 1	1.8	0.54	6.2
	<5 years	4 vs. 1	2.4	0.55	10.1
	5–10 years	2 vs. 1	1.7	0.67	4.1
	5–10 years	3 vs. 1	1.5	0.46	4.8
	5–10 years	4 vs. 1	1.5	0.36	6.1
	11–20 years	**2 vs. 1**	**2.7**	**1.1**	**6.5**
	11–20 years	3 vs. 1	2.4	0.78	7.4
	11–20 years	4 vs. 1	2.3	0.58	8.9
	>20 years	2 vs. 1	2.0	0.84	4.6
	>20 years	3 vs. 1	1.1	0.35	3.2
	>20 years	4 vs. 1	1.5	0.39	5.6
Country of practice (other = ref)	North America	2 vs. 1	0.9	0.71	1.2
	North America	**3 vs. 1**	**1.8**	**1.2**	**2.6**
	North America	4 vs. 1	1.5	0.95	2.3
Specialty (internist or primary care physician = ref)	Endocrinologist	2 vs. 1	2.0	0.68	5.9
	Endocrinologist	3 vs. 1	1.1	0.27	4.3
	Endocrinologist	4 vs. 1	1.4	0.25	7.5
	Surgeon	**2 vs. 1**	**3.9**	**1.2**	**13.0**
	Surgeon	3 vs. 1	1.1	0.22	5.2
	Surgeon	4 vs. 1	5.3	0.81	34.3
	Nuclear medicine physician	2 vs. 1	2.9	0.83	10.2
	Nuclear medicine physician	3 vs. 1	0.77	0.14	4.1
	Nuclear medicine physician	4 vs. 1	3.1	0.44	22.1
	Other	2 vs. 1	1.6	0.44	5.8
	Other	3 vs. 1	0.56	0.10	3.1
	Other	4 vs. 1	3.2	0.45	23.1

OR and CI indicated in bold font are significant.
Continuing LT4 = therapeutic group 1 (reference), Increasing LT4 = therapeutic group 2, Adding LT3 to same or reduced LT4 = group 3, Replacing LT4 with DTE or LT3 = group 4.

or small studies (24). It has become increasing common to involve patients in their own care and to incorporate joint physician-patient decision-making in the management of many conditions. It is possible that this management style, combined with increased attention to the possibilities of combination therapy in the media, social media, and patient support groups, has led to willingness to consider this therapy. Local prescribing patterns and interaction with pharmaceutical companies may also be influential.

If combination therapy is being more frequently prescribed, it is important to consider the potential risks, as well as the potential benefits. There is relatively little data available about the potential risks of combination therapy (35). This is, in part, because most studies of combination therapy are of short duration, with only one lasting a full year (5–17). One recent observational study did not identify an increased risk of atrial fibrillation or fractures with a median duration of LT3 therapy of 10.9 years (31). However, there was an increased risk of new prescriptions for antipsychotic medications and a trend for increased prescriptions of new anti-depressant medications with combination therapy.

When considering regional variations in prescribing combination therapy, the finding that combination therapy was considered more in North America than other regions was surprising, given that, in general, guidelines from North America recommend against such treatment (2, 4). It is possible that this simply reflects that most guidelines are widely disseminated throughout all geographic regions, as has been shown for ATA guidelines (36), and physicians may be particularly influenced not by guidelines from their region, but by the most recent guidelines. Unexplained variation in practice patterns has been

shown for other thyroid disorders, such as thyroid cancer (37) and subclinical hypothyroidism (38, 39).

There are several limitations of our study. The number of respondents at each of our time points was relatively small, represented a small percentage of the ATA membership, and was smaller at each successive time point. The patient scenarios were presented in the same order to all physicians, rather than in a random order, making it harder to correct for an effect of order. In addition, responses about the management of theoretical patients may not reflect what a physician would do when faced with a real patient. We also did not ask physicians whether they prescribed combination therapy to patients in their own practice or when they had last prescribed combination therapy in their own practice. We also do not have data available about the number of LT3 prescriptions actually written in the US or other countries over recent years or the availability of LT3 within various insurance prescription plans or within the various countries.

In summary, this study shows that some physicians are willing to prescribe combination therapy to patients with hypothyroidism. Prescribing patterns are affected by the characteristics of the patient and the characteristics of the physician, and these prescribing patterns may be changing over time. Given that little new evidence has accrued, this trend may be due to greater consideration of patient preferences. Better studies of physiologic doses of combination therapy that rigorously examine patient preferences, patient-reported outcomes, and quality of life are clearly needed. Without such studies, authors of future hypothyroidism treatment guidelines will face a substantial quandary as to how to weight patient preferences, and the physician prescribing patterns seen in this analysis, with the negative results of combination therapy trials.

AUTHOR CONTRIBUTIONS

JJ designed and conducted the study. NS and ET designed and analyzed the study.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2019.00031/full#supplementary-material

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The Swinging Pendulum in Treatment for Hypothyroidism: From (and Toward?) Combination Therapy

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Thyroid hormone replacement for hypothyroidism can be achieved via several approaches utilizing different preparations of thyroid hormones, T3, and/or T4. "Combination therapy" involves administration of both T3 and T4, and was technically the first treatment for hypothyroidism. It was lauded as a cure for the morbidity and mortality associated with myxedema, the most severe presentation of overt hypothyroidism. In the late nineteenth and the early Twentieth centuries, combination therapy *per se* could consist of thyroid gland transplant, or more commonly, consumption of desiccated animal thyroid, thyroid extract, or thyroglobulin. Combination therapy remained the mainstay of therapy for decades despite development of synthetic formulations of T4 and T3, because it was efficacious and cost effective. However, concerns emerged about the consistency and potency of desiccated thyroid hormone after cases were reported detailing either continued hypothyroidism or iatrogenic thyrotoxicosis. Development of the TSH radioimmunoassay and discovery of conversion of T4-to-T3 in humans led to a major transition in clinical practices away from combination therapy, to adoption of levothyroxine "monotherapy" as the standard of care. Levothyroxine monotherapy has a favorable safety profile and can effectively normalize the serum TSH, the most sensitive marker of hypothyroidism. Whether levothyroxine monotherapy restores thyroid hormone signaling within all tissues remains controversial. Evidence of persistent signs and symptoms of hypothyroidism during levothyroxine monotherapy at doses that normalize serum TSH is mounting. Hence, in the last decade there has been acknowledgment by all thyroid professional societies that there may be a role for the use of combination therapy; this represents a significant shift in the clinical practice guidelines. Further bolstering this trend are the recent findings that the Thr92AlaD2 polymorphism may reduce thyroid hormone signaling, resulting in localized and systemic hypothyroidism. This strengthens the hypothesis that treatment options could be personalized, taking into consideration genotypes and comorbidities. The development of long-acting formulations of liothyronine and continued advancements in development of thyroid regenerative therapy, may propel the field closer to adoption of a physiologic thyroid hormone replacement regimen with combination therapy.

Keywords: thyroid, levothyroxine, liothyronine, desiccated, history

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INTRODUCTION

Hypothyroidism is a prevalent condition, diagnosed in most cases by an elevation in serum TSH (1). While severe myxedema has been clinically recognized since the nineteenth century, the diagnosis of lower-grade hypothyroidism has not always been straightforward (2); early diagnostic attempts relied on parameters such as a slow basal metabolic rate (BMR), low serum protein-bound iodine (PBI), or even clinical responsiveness to thyroid preparations (3). In patients for whom the diagnosis has been secured, thyroid hormone replacement has been the mainstay of therapy for over a century (1, 3). Natural thyroid preparations, i.e., thyroid extract, desiccated thyroid, or thyroglobulin, were the first pharmacologic treatments while synthetic agents were introduced later and are the standard of care today (3). Despite major progress, there remains debate as to whether a universal approach is applicable to all patients and which agent constitutes the best thyroid hormone replacement.

Combination therapy via natural thyroid preparations remained the dominant therapeutic option for the better part of the twentieth century; dosages were adjusted to resolve symptoms and to normalize BMR/PBI (4–6). Yet with this regimen, thyrotoxic side effects were not uncommon (7). In the 1970's, the clinical approach to the hypothyroid patient changed markedly based on (i) the development of immunological assays to measure serum TSH as a more reliable biochemical index of thyroid activity (8), (ii) the accessible pricing of synthetic thyroid hormone formulations, and (iii) the discovery that in humans most circulating T3 is derived via extrathyroidal conversion of T4 (3, 9). These three factors led to a dramatic change in how hypothyroidism was diagnosed and treated, such that in the last 40 years (i) measurement of serum TSH has become the cornerstone of diagnosis and therapeutic monitoring, (ii) the replacement dosage of thyroid hormone has been substantially decreased, and (iii) "monotherapy" with levothyroxine (LT4) has become a universally accepted first-line approach given its excellent safety index. LT4 monotherapy establishes normalization of serum TSH levels and symptomatic remission for a majority of patients. Of course, the foundation for the success of this regimen is largely attributed to the physiologic action of the deiodinases (10); it is widely accepted that LT4 restores the pool of prohormone, T4, and the deiodinases regulate peripheral T3 production (11).

The efficacy of LT4 monotherapy has come into question as with this approach, 10–15% of patients express dissatisfaction due to residual symptoms of hypothyroidism (12, 13), and specifically cognitive impairment (14, 15). This might not have happened in the previous era given the much higher replacement doses of thyroid hormone used prior to the institution of the serum TSH radioimmunoassay (RIA) (3). In fact, when the dose of LT4 is adjusted to maintain a normal serum TSH, the ability of the deiodinases to appropriately regulate T3 availability has been challenged by the observation that about 15% of patients receiving LT4 alone fail to achieve normal serum T3 levels (15–17). The study of a number of animal models indicate that maintaining normal serum T3 levels is a biological priority (18). Although the clinical significance of relatively low serum T3 is not well-defined (1), there is evidence demonstrating that elevating serum T3 utilizing combination therapy can have improved symptomatology for some patients (19–22). Thus, given the high prevalence of hypothyroidism and the significant proportion of patients that remain symptomatic, this represents a target for improvement of the public health. The most recent treatment guidelines have been revised to acknowledge these gaps in the approach to hypothyroid patients (**Table 1**) (1, 26, 27).

There are new insights into the molecular mechanisms underlying the relatively lower serum T3 associated with LT4 monotherapy (28), namely that the hypothalamus exhibits altered D2 ubiquitination, explaining the inability of LT4 alone to normalize serum T3 levels (29). Only steady delivery of LT4 and LT3 in thyroidectomized rats fully normalizes serum and tissue T3 levels (30), as well as T3-dependent metabolic markers and gene expression profiles in this animal model (29). In humans, a large systematic review and meta-analysis recently showed that T3-dependent metabolic markers, such as total and LDL cholesterol, remain significantly higher in LT4-treated hypothyroid patients with normal serum TSH levels compared to healthy controls (31). A prevalent genetic polymorphism in the type 2 deiodinase, Thr92AlaD2, disrupts cellular morphology, has a prolonged half-life, is associated with ER stress and may exhibit decreased catalytic activity (32–34). Although further studies are needed to confirm these mechanisms and the clinical implications of a relatively low serum T3 need to be further defined, the available clinical evidence suggest that LT4 monotherapy may not represent a universal "replacement" for endogenous euthyroidism.

NEED FOR THYROID REPLACEMENT ESTABLISHED, TREATMENT STRATEGIES REFINED

Cases describing the clinical syndromes resulting from severe hypothyroidism, namely cretinism in children and myxedema in adults, were reported in the mid-nineteenth century (35–38) but were not initially connected with a deficiency from the thyroid gland (35, 39). The causal relationship was not understood until surgeons noted incident myxedema following total thyroidectomy (40, 41); milder symptoms consisting of a "*dull, listless, mental state*" were noted when only partial thyroidectomy was performed (42). By the late 1890's, its clinical features were well-described and its epidemiology better understood; myxedema could be sporadic, of insidious onset, occurred more commonly in women, and its prevalence variable by region (43).

Initial treatment strategies for hypothyroidism were largely insufficient, basically supportive and symptom-directed therapies: "*protection against cold, persistent use of hot baths with vigorous friction did much good;... The more favorable surroundings in hospital conferred temporary benefit on some cases, and removal to a mild and genial climate on others*" (44). The significant morbidity and mortality in the absence of efficacious treatment was clear, "*the progress of the disease is not readily affected by any remedy. The prognosis is altogether*

TABLE 1 | Trends in guidelines from professional societies.

Authors (year)	Professional Society	Rec #	Standard of care	Notes about combination therapy	Goal
Singer et al. (23)	ATA		LT4 "is the treatment of choice for the routine management of hypothyroidism"	• Chronic LT3 "not recommended" • DT "not necessarily contraindicated"	Normalization of serum TSH
Baskin et al. (24)	AACE/ATA		"all physicians will treat clinical hypothyroidism with" LT4	• "desiccated thyroid hormone, combinations of thyroid hormones, or triiodothyronine should not be used as replacement therapy" • "insufficient evidence is available to know which patients with hypothyroidism, if any, would be better treated with a combination of T4 plus T3 rather than with T4 alone"	Normalization of serum TSH
Garber et al. (25)	AACE/ATA	22.1	"Patients with hypothyroidism should be treated with L-thyroxine monotherapy"	• REC 22.2: "The evidence does not support using L-thyroxine and L-triiodothyronine combinations to treat hypothyroidism." • REC 22.4: "There is no evidence to support using desiccated thyroid hormone in preference to L-thyroxine monotherapy in the treatment of hypothyroidism and therefore desiccated thyroid hormone should not be used for the treatment of hypothyroidism."	Normalization of serum TSH
Wiersinga et al. (26)	ETA	1, 2	Acknowledgment that some LT4-treated patients with normal serum TSH may have persistent symptoms	Rec 7: "L-T4 + L-T3 combination therapy might be considered as an experimental approach in compliant L-T4-treated hypothyroid patients who have persistent complaints despite serum TSH values within the reference range"	"The goal of…combination therapy is to resolve persistent complaints despite a normal TSH in L-T4-treated hypothyroid patients. In an attempt to realize this goal, it is assumed that…a euthyroid state simultaneously in all tissues of hypothyroid patients is present if serum TSH, free T4, free T3, and free T4:free T3 ratio are all within the reference range."
Jonklaas et al. (1)	ATA	1a	"Levothyroxine is recommended as the preparation of choice for the treatment of hypothyroidism"	Rec 13c: "For patients with primary hypothyroidism who feel unwell on levothyroxine therapy alone…there is currently insufficient evidence to support the routine use of a trial of a combination of levothyroxine and liothyronine therapy…due to uncertainty in long-term risk benefit ratio of the treatment and uncertainty as to the optimal definition of a successful trial to guide clinical decision-making."	Rec 1b: LT4 treatment goals include "(i) to provide resolution of the patients' symptoms and hypothyroid signs, including biological and physiologic markers of hypothyroidism, (ii) to achieve normalization of serum thyrotropin with improvement in thyroid hormone concentrations"
Okosieme et al. (27)	BTF	5	"L-T4 remains the treatment of choice in hypothyroidism with the aim of therapy being to restore physical and psychological well-being while maintaining normal laboratory reference range serum TSH levels"	• Rec 10: "L-T4/L-T3 combination therapy in patients with hypothyroidism should not be used routinely" • Rec 12: "If a decision is made to embark on a trial of L-T4/L-T3 combination therapy in patients who have unambiguously not benefited from L-T4, then this should be reached following an open and balanced discussion of the uncertain benefits, likely risks of over-replacement and lack of long-term safety data."	Normalization of serum TSH

AACE, American Academy of Clinical Endocrinologists; ATA, American Thyroid Association; BTF, British Thyroid Association; ETA, European Thyroid Association; LT4/L-T4, levothyroxine; LT3/L-T3, liothyronine; DT, desiccated thyroid; TSH, thyroid-stimulating hormone.

unfavorable," (45) and thus the need to "replace" the thyroid was established. Thyroid transplant (46–50), seemingly the most divergent from contemporary approaches, had some early successes as many patients had improvement after receiving animal (sheep or goat, preferably pregnant) or human thyroid glands taken from patients with Graves' disease or goiter. Grafts were typically transplanted into the tibia or the abdominal cavity. For many patients, symptoms recurred and the procedure was repeated in some up to four times (51). Due to the rapid and transient improvement observed, "*too soon, therefore for the gland to have become vascularized and functionally active in its new situation*" (44), it was hypothesized that symptoms improved by absorption of the secretions of the donor gland (47, 52). Whereas, thyroid transplant was likely to provide uncertain quantities of the two hormones, T4 and T3, it could nevertheless be considered to be the earliest examples of a form of "combination therapy."

Trials of the first pharmacologic strategies included other combination therapies: intravenous/subcutaneous administration of thyroid extract was utilized by Murray to treat myxedema (44, 53–55), per oral thyroid extract (56, 57), or the consumption of raw or cooked thyroid gland (55, 58, 59). These strategies saw remarkable successes, "*the results are perfectly marvelous*" (60). Oral thyroid replacement strategies won favor as their successes were undeniable and without the morbidities and relapse rates associated with transplant. However, it was noted early on that there could be side effects of treatment: "*thyroid gland…is responsible for distressing and even alarming symptoms,*" but the details were not fully described (61). Progress toward a modern thyroid transplant treatment modality is ongoing given that functional thyroid tissue can be generated from stem cells by over-expression of the thyroid transcription factors (62–64), in which case the field would have come full circle (65).

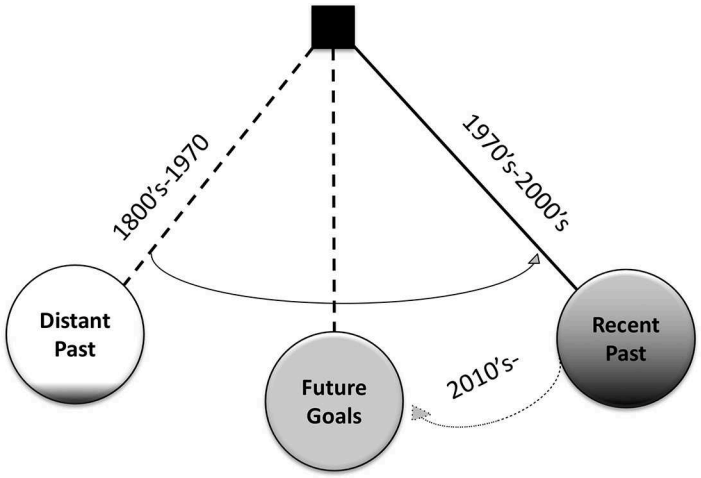
Thyroxine was crystallized in 1915 by Kendall (66), its chemical structure identified (67), and was administered successfully as an IV therapy by 1925 (68). This provided the basis for the development of synthetic LT4 (69, 70), which was shown to be efficacious in the treatment of myxedema (71) and in patients who failed to respond to desiccated thyroid treatment where clinical response was defined as BMR and restoration of ovulation/fertility (72). In 1952, serum T3 was discovered by Gross and Pitt-Rivers (73, 74). Serum PBI emerged as a diagnostic test and therapeutic marker, reflecting the combined amounts of circulating, protein-bound, T4 and T3. In the era prior to the availability of the TSH assay, this was the most specific diagnostic tool (75). However, PBI was limited in terms of monitoring a response to treatment as the "*concentration of the PBI associated with restoration of a normal metabolic state depends upon the particular thyroid hormone employed*" (76). For example, LT3 was reported as correcting BMR without much increase in PBI (77), whereas LT4 increased PBI sometimes to above the upper limit of the normal range (78), and combination LT4 + LT3 and desiccated thyroid had the advantage of normalizing PBI (79).

As such, mixtures of LT4 and LT3 administered concomitantly were proposed and developed, "*the ideal thyroid hormone preparation should combine* [LT4 and LT3] *in physiologic proportions to simulate the metabolic effects of endogenous thyroid hormone secretions*" (80). These investigators concluded that a mixture of 175 mcg LT4:50mcg LT3 was ideal because it optimized both BMR and PBI (80), but other investigators proposed ratios on the order of about 9:1 (81). Thus, despite the development of LT4, combination therapy via LT4 + LT3 or desiccated thyroid was still the preferred regimen (3).

Clinical trials were designed to assess efficacy and dose equivalency between the multiple forms of thyroid hormone replacement. Importantly, (i) these were not designed as superiority trials, (ii) outcomes assessed included normalization of PBI and/or BMR, and (iii) doses were dramatically higher than used today (3). Therefore, it is difficult to determine whether any thyrotoxic side effects were related to the type of the agent used or a consequence of its high dosage. For example, in studies utilizing doses of LT3 75–100 mcg/day, angina and congestive heart failure were observed (82); in another trial, palpitations, irritability, nervousness, dizziness, tremor, and perspiration were observed on LT4 (80 mcg) plus LT3 (20 mcg) daily (83).

Despite these concerns, it was noted that these thyrotoxic side effects were typically remediable by simple reduction in dosage (82, 84), so combination therapy, usually by desiccated thyroid, remained the preparation of choice (85) through the mid-1970's for the treatment of hypothyroidism (3). This preference was reinforced by the unique ability of desiccated thyroid to reproduce a normal PBI as compared with LT3 or LT4 monotherapies, making biochemical monitoring more straightforward (**Figure 1**) (7, 80). In 1965, approximately four out of every five prescriptions for thyroid hormone were for natural preparations in the US (86).

Prominent manufacturers of natural thyroid products including both desiccated thyroid (87) and thyroglobulin (88), boasted about their "*double standardization*" (87) methods to ensure "*unvarying metabolic activity*" (88) between batches; this included (i) chemical assessment of iodine content to adhere to the standards of the British or United States Pharmacopeia (BP or USP) as well as (ii) biologic activity assessed by change in oxygen consumption in treated guinea pigs (89) or its ability to reduce the size of animal goiters (90). Despite these efforts, clinicians remained rightfully concerned regarding inconsistencies in the potency of these tablets (91). Even as early as 1911, physicians understood that there was variability within natural thyroid preparations, "*there are probably some [preparations of thyroid] on the market that are inactive. It is only natural that the properties and activity of the gland should vary in different animals, according to their age and sex, and probably even according to their pasturage*" (92). Despite adherence to iodine content standards, some batches had varying potency (76), such that tablets contained nearly double potency, and others had almost no detectable metabolic activity (93). Also, humidity limited the shelf-life of desiccated tablets (84). There were reports of patients failing to respond to desiccated thyroid altogether as their tablets contained no active thyroid hormone (94–96). This led to claims that desiccated thyroid was dangerous and "*that its manufacture be abolished*" (97); it became viewed by many as "*obsolete*" as it "*possesses no uniquely desirable properties and should, therefore be retired to the place that it has earned in*



Standard of care	Combination therapy (thyroid transplant, desiccated thyroid, thyroid extract)	*Physiologic* combination therapy (based on genotype?)	LT4 monotherapy
Monitoring	Clinical/BMR/PBI	*Physiologic* T4:T3 ratio? T3-dependent markers? (serum TSH, BMR, serum lipids, other tissue-specific markers) Role of subjective symptoms?	Serum TSH
Prevalent clinical sequelae	Thyrotoxicosis	Goal: Euthyroidism	Hypothyroidism

FIGURE 1 | Historical Trends in the Treatment of Hypothyroidism. In the late 1800's–mid 1900's, combination therapy via thyroid transplant, thyroid feeding, thyroid extracts, thyroglobulin, or desiccated thyroid was preferred. Treatment was monitored by clinical response, basal metabolic rate (BMR), and/or serum protein-bound iodine (PBI). Thyrotoxic symptoms were prevalent in early clinical trials. In juxtaposition, levothyroxine (LT4) monotherapy to achieve normal serum TSH levels was adopted as the standard of care in the 1970's. It has become more recognized that patients on this regimen can have residual signs and symptoms of hypothyroidism. Thus, underscoring the need for new, physiologic thyroid replacement regimens with the goal to restore thyroid hormone signaling within all tissues. With the recognition that genetic polymorphisms may play a significant clinical role, personalized medicine will likely be integrated into future clinical trials and treatment regimens.

medical history" (98). It was not until 1985 that the revision of the USP standard from iodine content to T3/T4 content established stable potency (86).

TRANSITION AWAY FROM COMBINATION THERAPY

Despite growing discontent with variable potency of natural thyroid products (93), as well as lowering in cost of LT4 such that the two treatments were approximately equivalent (99), physicians hesitated to use LT4 monotherapy, concerned that it could result in a relative T3 deficiency (84, 91). However, the landmark discovery of peripheral T4-to-T3 conversion in athyreotic humans by Braverman et al. obviated this concern (9) and provided the foundation for the hypothesis that LT4 could replace prohormone pool and the deiodinases would regulate availability of active T3 (11). This discovery had a major influence on the prescribing practices of physicians such that within about a decade there was a major transition toward LT4 as the first-line therapy in hypothyroidism (3, 86, 90).

The TSH RIA was developed by Utiger almost simultaneously (8). Clinicians were able to titrate therapy to achieve a serum TSH within the normal range as a specific marker of thyroid hormone replacement adequacy (100–102). This came with the caveat that early TSH assays were not able to distinguish between normal and low serum TSH levels (103). Thus, patients treated to a suppressed TSH (normal for that method) could only be differentiated through employment of a second test, the thyrotropin-releasing hormone (TRH) stimulation test, which could identify over-treated patients (suppressed TSH) by their subnormal response to exogenous TRH (104). For patients that were once treated with doses that normalized their symptoms, BMR, or PBI, the utilization of serum TSH (associated with the TRH stimulation test) revealed such doses to be typically supratherapeutic (14, 103, 105, 106). Whereas, prior to the institution of the TSH assay, typical maintenance doses of LT4 were in the 200–500 mcg/day range, doses were now typically closer to 100–150 mcg/day (3, 103, 105, 106).

Soon thereafter was the development of RIAs for measurement of serum T3 (107, 108) and T4 (109). With the availability of these assays, it was observed that LT4 could normalize both T4 and T3 levels at the expense of a high T4/T3

ratio. LT3, desiccated thyroid, thyroglobulin, and LT4 + LT3 combination all typically resulted in low or low-normal T4 values with usually elevated T3 levels (90). In particular, it was noted that desiccated thyroid resulted in a T3 peak occurring about 2–5 h after administration that corresponds to thyrotoxic symptoms in some patients (99). That a single daily dose of oral LT4 resulted in nearly seemingly physiologic, stable blood levels of T4 and T3 throughout the day (107) was understood to be a result of a steady rate of conversion of T4-to-T3 (110, 111). In less than a decade after discovery of peripheral T4-to-T3 conversion and with implementation of RIAs to specifically quantitate serum TSH, T4, and T3, normalization of TSH with LT4 became the new standard of care (**Figure 1**) (3, 112). These findings left many clinicians advocating not only for LT4 to be the first-line therapy, but that patients previously treated with desiccated thyroid be transitioned to LT4 (99).

DEFINING "EUTHYROIDISM" AND REVISITING COMBINATION THERAPY

Following the transition to LT4 monotherapy and reduction in replacement dose to achieve a normal TSH, clinicians noted several important differences in the ability of this regimen to normalize markers of hypothyroidism such as BMR, serum cholesterol, and patient satisfaction (3). In many LT4-treated hypothyroid patients with a normal TSH, the BMR remained at about −10% of that of normal controls (113). Whereas, LT4 treatment at doses that normalize BMR, can suppress the serum TSH (90, 103, 105, 106, 114). Recent investigations have confirmed that energy expenditure is only normalized in LT4 treatment at doses that suppress the serum TSH (115). Another study found that energy expenditure does not differ between groups treated with LT4 doses to result in either high-normal or low-normal serum TSH levels (116). Hypothyroidism is a secondary cause of dyslipidemia, typically manifesting in elevation of LDL and total cholesterol levels (31), however, it was noted that normalization of LDL in LT4-treated hypothyroid patients can require TSH-suppressive doses (117, 118). Complaints from dissatisfied patients treated with LT4 monotherapy at doses to normalize the serum TSH were often dismissed as unrelated to their thyroid condition (119), or attributed to non-compliance (120), as symptoms are non-specific and can overlap with other common conditions including menopause, depression, and chronic fatigue syndrome (2). However, LT4-treated patients display significant impairment in psychological well-being compared to controls of similar age and sex (14). To assess whether this was a result of trends toward lower doses of LT4, measures of well-being were tracked on various doses and it was found that the highest well-being is achieved at doses resulting in a suppressed TSH (121). However, such findings were not always reproducible (122). Indeed, it has been shown in a large population study that LT4-treated patients exhibit higher BMIs and take more statins and anti-depressants than TSH-matched, healthy controls (15); this association could have been impacted by confounding and thus further investigation is indicated to confirm these results (15). Thus, in LT4 monotherapy, defining euthyroidism as normal serum TSH has flaws as other clinical parameters may not be normalized.

Overtreatment associated with a low serum TSH, is associated with increased cardiovascular and skeletal risks (1), thus in the current guidelines a goal of therapy remains achievement of normal serum TSH levels (**Table 1**) (1, 26, 27). A small study found that patients perceived that their physicians were overly reliant on serum TSH levels and that this was a barrier to them receiving optimal care (123). Prescribing patterns have changed such that serum TSH level at time of initial treatment has been decreasing (124, 125) yet this may not improve quality of life or thyroid-related symptoms (126). Thus, reconciliation between optimization of patient outcomes without the increased risks of overtreatment remains a unique challenge in the field.

It should be noted that assessment and interpretation of serum T3 levels presents significant limitations as well due to (i) the difficulties accurately measuring serum free T3 with standard clinical lab assays (18), (ii) the fact that serum T3 levels may not fully represent intracellular T3 due to intracellular deiodination (10), and (iii) other non-thyroidal illnesses are known to result in low serum T3 (1, 18). In a study of 42 patients, assessment of serum T3 at baseline and during combination therapy did not predict positive, symptomatic response (127). Thus, the clinical utility of serum T3 measurements is unknown (1).

In a clinical trial of combination therapy with LT4 + LT3 to establish normal serum TSHs, there was improvement in psychological parameters (19). In another study comparing LT4 monotherapy to desiccated thyroid, in which both groups had a normal TSH, 48% of patients preferred desiccated thyroid over LT4 monotherapy (18.6% preferred LT4) and those patients preferring dessicated thyroid also experienced about 4 pound weight loss over the 16 week treatment period (21). Indeed, many clinical trials show subjective "preference" for combination therapy without positive objective results when utilizing quality of life and/or thyroid-specific questionnaires (19, 21, 128, 129). This suggests that these questionnaires may not be capturing the parameters improved by combination therapy, and opens yet another path for further research. Benefits with combination therapy have not been reproduced in all populations, and many studies fail to demonstrate superiority of combination therapy (1, 14). This may be related to the pharmacologic properties of available oral LT3 preparations. There are theoretical concerns about adverse events with LT3 treatment, but in one observational study over 17 years, there were no increased cardiovascular or skeletal risks (130).

Modern professional societies have synthesized their best practice guidelines for hypothyroidism. These guidelines have been evolving away from a universal approach with LT4 monotherapy (23, 24, 131) and toward an approach that accepts a therapeutic trial of combination therapy for select patients (**Table 1**) (1, 26, 27). In 1995, the American Thyroid Association (ATA) recommended LT4 monotherapy, and recommended against LT3 due to risk of iatrogenic thyrotoxicosis (23). In their conjoint guideline, the ATA and the American Association of Clinical Endocrinologists (AACE) stated that "*all physicians*

will treat clinical hypothyroidism with levothyroxine" (24), and also recommended against combination therapy (**Table 1**). These recommendations were similarly upheld in the ATA/AACE guidelines in 2012 (131). The 1995, 2002, and 2012 guidelines all recommended normalization of serum TSH as the treatment goal (**Table 1**; **Figure 1**) (23, 24, 131).

However, in 2012, the European Thyroid Association (ETA) published guidelines (26) in contrast to those of ATA/AACE (131). These ETA guidelines acknowledged that some LT4-treated patients with normal serum TSHs may have persistent symptoms based on increased verbalization from patient advocacy groups and supportive evidence from some clinical trials (26, 128). These guidelines also clearly documented hypotheses for treatment dissatisfaction among hypothyroid individuals: causes related to disease chronicity, associated autoimmune diseases, thyroid autoimmunity, inadequate LT4 dosing, and inadequacy of LT4 treatment modality (26). The acknowledgment of dissatisfaction among a significant proportion of individuals and documentation of possible etiologies to stimulate future research (26), seem to be in stark contrast to the previous paradigm of universal LT4 monotherapy (**Table 1**) (23, 24, 131). The ETA offered a second-line approach for these symptomatic individuals: using LT4 + LT3 combination therapy for select individuals in "carefully executed" therapeutic trials. The ATA then built upon these recommendations given the new evidence that polymorphisms in the deiodinases can be associated with differences in serum thyroid hormone levels (132) and acknowledgment that some LT4-treated individuals have relatively low serum T3 concentrations (1, 16, 17, 133–135). The ATA did note that there were inconsistent findings from clinical trials of combination therapy, thus superiority of combination therapy had not been established (1). Goals of therapy included normalization of serum TSH and "*to provide resolution of the patients' symptoms and hypothyroid signs, including biological and physiologic markers of hypothyroidism*" (1). Although these markers were not well-defined, this represented a significant shift compared to prior ATA guidelines (**Table 1**) (23, 131). The British Thyroid Foundation likewise recommended therapeutic trial of LT4 + LT3 combination therapy to "*restore physical and psychological well-being*" (27). Despite consensus from these societies that LT4 monotherapy remain as first-line, a recent survey found that at least 58% of clinicians would prescribe a trial of combination therapy for specific clinical scenarios in which LT4-treated patients with normal serum TSH exhibited residual symptoms (136).

NEW EVIDENCE MAY JUSTIFY COMBINATION THERAPY

The importance of investigating the benefits associated with combination therapy in humans is highlighted by findings in an animal model of hypothyroidism. As in humans, LT4 monotherapy for athyreotic rats results in a high T4:T3 ratio at doses sufficient to normalize serum TSH levels (29). Yet, the brain, liver and skeletal muscle tissues of these LT4-treated animals exhibit markers of localized hypothyroidism (29), likely due the inability of LT4 monotherapy to restore tissue levels of T3 (30). This occurs as a direct consequence of the relatively high T4 concentration in these tissues: D2 downregulation is T4-mediated. In the hypothalamus, as a result of localized reduction in D2 ubiquitination, there is increased sensitivity to T4 levels, explaining the ability of the TSH to be normalized despite relatively lower levels of serum T3. Thus, only combination therapy with stable release LT4 + LT3 normalized all parameters of thyroid hormone homoeostasis (29) including serum and tissue T3 levels in rodents (30). In humans, LT4 monotherapy results in a high T4:T3 ratio (15, 16, 120, 137), thus underscoring the importance in establishing its clinical significance (1, 18, 31, 138). A large systematic review and meta-analysis of T3-dependent markers in hypothyroid humans treated with LT4 monotherapy, showed that LDL (3.31 ± 1.64 mg/dL) and total cholesterol (9.60 ± 3.55 mg/dL) remain higher in LT4-treated patients than healthy controls, despite normalization of serum TSH (31). The clinical significance of this difference in serum cholesterol remains to be determined, however this may justify well-designed clinical trials of combination therapy utilizing tissue-specific markers of thyroid status as outcome measures.

One factor that has been associated with response to combination therapy in multiple clinical trials is the Thr92Ala polymorphism in the type 2 deiodinase, where carriers can exhibit improved quality of life measures and preference for combination therapy (20, 22). This has led to the logical hypothesis that Thr92AlaD2 could be associated with localized and/or systemic hypothyroidism, yet results from clinical trials have not been consistently supportive (139–141). Multiple groups have demonstrated normal *in vitro* Thr92AlaD2 enzyme kinetics (142, 143), but other groups have found evidence of reduced enzymatic activity at the cellular and organism level (144). The Thr92AlaD2 protein has been found to disturb cellular physiology: it had a longer half-life, localized in the Golgi apparatus and significantly alter the transcriptome while stably expressed *in vitro* (33). In the same study, it was demonstrated that in the human temporal pole the transcriptome was similarly altered, resulting in a proposed 81-gene fingerprint of Thr92AlaD2 expression (33). An unexpected finding was that this transcriptome from human temporal pole samples shared expression patterns found in neurodegenerative diseases. Indeed, in a large cohort, African American carriers of Thr92AlaD2 exhibited about 30% greater risk of developing Alzheimer's disease (32), suggesting that the study of Thr92AlaD2 transcends the thyroid field. In a novel animal model of Thr92AlaD2 expression, there was evidence of ER stress and neurocognitive dysfunction; with administration of LT3 to animals with intact endogenous thyroids, the phenotype improved, bolstering support for the positive findings in many clinical trials (34). As the molecular basis for the Thr92AlaD2 observations is better characterized, it remains to be confirmed whether hypothyroid carriers may benefit from individualized therapies. If so, then the notion of personalized medicine, based on genotype, may define the future of management in hypothyroidism (3).

A slow-release oral form of LT3 was recently developed and applied in hypothyroid rats where it was found to provide stable, normal serum T3 levels (145). Results from human trials with this agent have yet to be determined, but this provides great hope that future high quality, randomized, controlled

clinical trials will establish whether steady-dose LT3 + LT4 combination therapy is superior to LT4 monotherapy in terms of its ability to normalize all parameters of thyroid hormone homoeostasis, including tissue markers, mood, and cognition. Of course such trials would need to evaluate whether patients with the Thr92AlaD2 polymorphism, and other polymorphisms that could be relevant in thyroid hormone signaling, respond uniquely to treatments.

In conclusion, whereas combination therapy once dominated, this trend was largely abandoned in the 1970's due to evidence of iatrogenic thyrotoxicosis and concerns of consistency. In addition, a consequence of the availability of sensitive TSH assays was a dramatic reduction in thyroid hormone replacement dosage. Discovery of peripheral T4-to-T3 conversion provided initial physiologic justification for LT4 monotherapy. Clinical practice trended away from natural thyroid preparations and toward LT4 monotherapy given at doses to normalize the serum TSH. This transition was associated with the emergence of a population of patients with residual signs and symptoms of hypothyroidism and relatively lower serum T3 levels, despite normalization of serum TSH levels. New evidence that genetic polymorphisms may affect thyroid hormone signaling may substantiate objective evidence of residual localized and/or systemic hypothyroidism in a proportion of the population. The development of long acting formulations of LT3 to result in stable serum T3 levels may bolster development of a physiologic thyroid hormone replacement regimen to better mimic endogenous euthyroidism.

AUTHOR CONTRIBUTIONS

EM performed the literature review, drafted the manuscript, table, and figure, and edited the manuscript. AB contributed to hypothesis generation and edited the manuscript.

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SYSTEMATIC REVIEW
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A Systematic Review and Meta-Analysis of Patient Preferences for Combination Thyroid Hormone Treatment for Hypothyroidism

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Background: The standard of care in management of hypothyroidism is treatment with levothyroxine (L-T4). Sometimes patients are dissatisfied with L-T4 and the combination of levo-triiodothyronine (L-T3) with L-T4 is considered.

Methods: We performed a systematic review and meta-analysis of blinded randomized controlled trials (RCTs), reporting how often hypothyroid patients prefer combination L-T3/L-T4 treatment to L-T4 alone. We also explored for explanatory factors for combination therapy preference in sensitivity analyses examining trial, patient, and disease characteristics. Potential dose-response relationships were explored using meta-regression analyses. We searched 9 electronic databases (from inception until February, 2019), supplemented with a hand-search. Two reviewers independently screened abstracts and citations and reviewed full-text papers, with consensus achieved on the included studies. Two reviewers independently critically appraised the quality of included studies and abstracted the data. Random effects meta-analyses were reported for the percentage of patients preferring combination L-T3/T-T4 therapy over L-T4 alone. A binomial distribution of choices (i.e., preference of combination therapy or no preference for combination therapy) was assumed.

Results: We included 7 blinded RCTs including 348 hypothyroid individuals in the primary meta-analysis. The pooled prevalence rate for preference of combination therapy over L-T4 was 46.2% (95% confidence interval 40.2%, 52.4%) ($p = 0.231$ for the difference from chance). There was no significant statistical heterogeneity among study results (Q = 7.32, degrees of freedom = 6, $p = 0.293$, $I^2 = 18.0\%$). In sensitivity analyses, combination treatment preference was explained in part by treatment effects on TSH concentration, mood and symptoms, but not quality of life nor body weight. In a secondary dose-response meta-regression analyses, a statistically significant association of treatment preference was identified for total daily L-T3 dose, but not L-T3:L-T4 dose ratio.

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Conclusions: In conclusion, in RCTs in which patients and investigators were blinded to treatment allocation, approximately half of participants reported preferring combination L-T3 and L-T4 therapy compared to L-T4 alone; this finding was not distinguishable from chance. An observed potential positive L-T3 dose effect on treatment preference deserves further study, with careful consideration of thyroid biochemical indices and patient reported outcomes.

Keywords: hypothyroidism, thyroid hormone, levothyroxine, triiodothyronine, systematic review, meta-analysis, randomized controlled trials

INTRODUCTION

Internationally, levothyroxine (L-T4) treatment is the established first choice as a standard of care in the management of hypothyroidism (1–7). However, some patients are dissatisfied with L-T4 standard care treatment (8). Factors contributing to dissatisfaction with thyroid hormone treatment include persistent hypothyroid symptoms, such as excess weight, fatigue, mood problems, or memory/cognitive concerns (8). In the clinical context of persistent symptoms after achieving a normalized thyroid stimulating hormone (TSH) concentration on L-T4, after excluding or managing other potential causes of symptoms, patients and clinicians sometimes consider utilizing alternative thyroid hormone preparations, such as combination therapy using L-T4 and levo-triiodothyronine (L-T3). The rationale for this approach would be normalizing potentially low tissue T3 levels, which are not readily measurable. We conducted a systematic review and meta-analysis, examining how often hypothyroid patients prefer L-T3/L-T4 combination therapy over L-T4 monotherapy. In order to minimize the risk of bias, we restricted our review to blinded randomized controlled trials (RCTs). We also explored for explanatory factors relating to patient preferences.

METHODS

Our systematic review was registered (PROSPERO CRD42019123920). We included blinded RCTs, examining how often hypothyroid adult patients prefer combination L-T3/L-T4 therapy, compared to the standard of care of L-T4 monotherapy. Trial settings were restricted to be ambulatory outpatient clinics (i.e., not hospitalized patients) and participants were required to be aged $\geq$ 18 years of age, with hypothyroidism of any etiology. Trials were required to report some level of blinding, including blinding of study participants. All studies were required to have measured thyroid function in study participants using a thyroid stimulating hormone (TSH) measurement. Studies focusing on desiccated thyroid hormone were excluded. Due to limited resources for translation, only English language studies were included. For overlapping or duplicate studies reporting the same primary outcome, the largest study was included. As we expected strong reader interest in factors explaining patient preferences, we separately abstracted data from secondary explanatory analyses (such as deiodinase polymorphism status), which would typically be published in subsequent publications from the original studies. The explanatory data was not included in the meta-analysis of combination therapy preference rate.

An experienced library information specialist (RF) executed a comprehensive search strategy from inception to February 2019 in the following databases: MEDLINE, Ovid MEDLINE Epub Ahead of Print and In-Process and Other Non-Indexed Citations, Embase Classic + Embase, "Cochrane Central Register of Controlled Trials," "Cochrane Database of Systematic Reviews," Emcare, and PsycInfo all from the OvidSP platform; Web of Science from the Clarivate Analytic, and ClincalTrials.gov. We limited our search to adults (age $\geq$ 18 years) and the following types of studies: randomized controlled trials, systematic reviews, and meta-analyses. There was no language restriction on the search. Where available, both controlled vocabulary terms ("exploded" where applicable), and text words were used to identify as many relevant results as possible (**Supplementary Data**). We supplemented the electronic search by cross-referencing included papers, relevant sections of clinical practice guidelines, relevant systematic and narrative reviews, as well as reviewing the personal files of one of the authors who had participated in development of a hypothyroidism clinical practice guideline (AMS).

Two investigators (AA and AMS) independently, in duplicate, screened citations from the electronic search, reviewed full-text papers for inclusion, critically appraised the quality included studies, and abstracted the data. Consensus was achieved for inclusion of papers and abstracted data by discussion of reviewers; a third reviewer/clinical content expert (SE) was consulted in the event of any discrepancies that could not otherwise be resolved by reviewer discussion. We contacted the corresponding authors of original studies if there were questions relating to potential eligibility or results of studies or the results. The risk of bias of included trials was evaluated using the most current Risk of Bias evaluation tool developed by the Cochrane Collaboration (ROB 2.0) (9). The systematic review was reported according to PRISMA standards (10).

Descriptive data were summarized as numbers and percentages for categorical data and means or medians and standard deviations or ranges for continuous data. We performed random effects meta-analyses, estimating the percentage (with 95% confidence intervals, CI) of patients preferring combination L-T4 with L-T3 (any dose) over L-T4 monotherapy (Comprehensive Meta-Analysis software, version 2.0). A binomial distribution for preferring (or not preferring) combination therapy was assumed, such that a significant

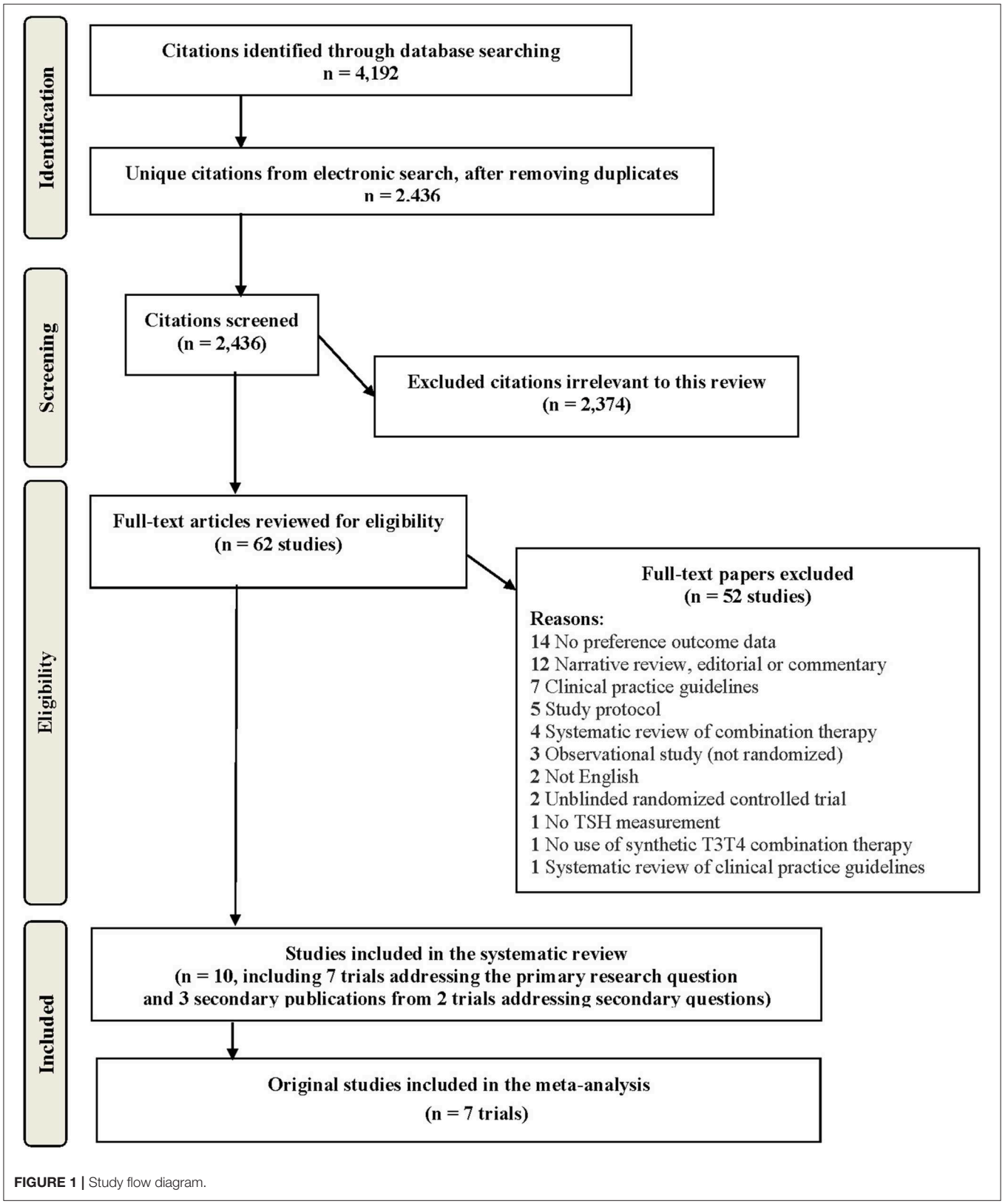

FIGURE 1 | Study flow diagram.

preference in combination therapy (beyond chance) would be defined by the lower limit of the 95% CI exceeding 50%. Individuals who preferred L-T4 or those who had no treatment preference, were judged to not prefer combination therapy. For the primary meta-analysis, treatment preference was evaluated only in individuals who had been exposed to L-T3 in a random fashion during the trial (i.e., individuals not receiving L-T3 at any point in the trial or those assigned L-T3 in non-random fashion, were not included). We evaluated for statistical heterogeneity in the meta-analysis using a Cochrane's Q (chi-squared) test (11) and an I^2 estimate (12). We planned to evaluate for potential publication bias using a funnel plot (13), assuming at least 10 studies were included in the meta-analysis (for meaningful interpretation). We planned to explore for sources of heterogeneity of combination therapy treatment preference by performing the following sensitivity analyses (examining for difference in treatment effects among studies according to study characteristics): (a) study characteristics (study quality, study drug treatment duration [3 months or less, or longer than 3 months]), (b) participant characteristics (mean or median age $<$ 50 years or $\geq$ 50 years], sex [inclusion of any men], molecular characteristics, treatment (frequency of daily treatment dosing), other treatment effects (differential changes between groups on TSH concentration, quality of life, mood, psychological outcomes, or body weight), and disease characteristics (etiology of hypothyroidism, i.e., inclusion of any patients who had thyroidectomy or radioactive iodine treatment as an etiology for hypothyroidism). Fixed effects univariate meta-regression was performed to investigate for any dose-effect on thyroid hormone treatment preference, relating to study- and study subgroup-specific combination therapy dose, specifically: L-T3 total daily dose (ug) and L-T3:L-T4 ratio of daily dose. These variables were calculated for the hypothetical scenario of an individual receiving a baseline L-T4 dose of 100 ug daily, in order to account for differences in calculation of combination therapy dose among trials (i.e., dose ratios or fixed dose substitutions). We utilized the L-T3:L-T4 ratio (and not vice versa), to enable inclusion of data from individuals receiving a dosage of 0 ug of L-T3 (in the case of individuals randomized to an L-T4 arm, in a parallel design trial). Thus, in the case of parallel design randomized controlled trials, the L-T4 arm data (not exposed to combination therapy) was not included in the primary analysis of prevalence of combination therapy preference but would be eligible for inclusion in the secondary meta-regression dose analysis, where the outcome was study drug treatment preference (i.e., placebo compared to pre-trial L-T4 use in the case of parallel design trials or combination therapy compared to intra-trial L-T4 use for cross-over trials). We defined statistical significance of all analyses at an alpha level of 0.05; however, in examining for heterogeneity using Cochrane's Q test, we set that alpha level at 0.10 (11).

RESULTS

As detailed in our study flow diagram (**Figure 1**), we retrieved a total of 4,192 citations from our electronic searches, ultimately yielding 2,436 unique citations after removing any duplicates. References from the hand search were all included in the electronic database searches, so the hand search yielded no additional relevant papers. We reviewed 62 full-text papers for eligibility and there were 7 trials included in the systematic review and meta-analysis (14–20). Relevant data on secondary subgroup analyses relating to molecular data were reported in two additional publications for the trial of Appelhof et al. [original publication (14), secondary publications (21, 22)] as well as Nygaard et al. [original publication (18), secondary publication (23)]. The reviewed full-text papers excluded from this review, and the reasons for exclusion are shown in the **Supplementary Table 1**. We excluded some RCTs comparing combination therapy compared to levothyroxine for the following reasons: (a) no data on patient preference—Clyde et al. (24), Kaminski et al. (25), Saravanan et al. (26), Sawka et al. (27), Siegmund et al. (28), Valizadeh et al. (29), (b) no blinding—Fadayev et al. (30), and (c) no TSH measurement (so safety and appropriateness of thyroid hormone dosing could not be established)—Smith et al. (31).

A summary of the details of the RCTs included in both the systematic review and meta-analysis is shown in **Table 1**. Of the included studies, five were conducted in Europe (14–18), one was conducted in the United States (19), and one was conducted in Australia (20). The number of participants randomized ranged from 13 to 141 hypothyroid patients (14–20). The majority of participants in the studies were female, with two studies recruiting only females (16, 17). Furthermore, the mean age of participants was younger than 50 years in all of the studies (14–20). The etiology of hypothyroidism was autoimmune primary hypothyroidism in the majority of participants in 5/7 studies (14, 17–20). None of the studies included patients with secondary hypothyroidism due to hypothalamic/pituitary disease. Of the 6 studies reporting on recruitment setting (14–16, 18–20), 5 reported recruiting participants in ambulatory Endocrinology clinics (15, 16, 18–20), and one reported recruiting participants from primary care practices (14). One study used a parallel design (14), whereas the other 6 studies utilized a cross-over design (15–20). Pre-trial L-T4 dose was required to be stable for at least 2 months in one study (20), 3 months in 2 studies (15, 19), 6 months in 2 studies (14, 18), and 1 year in one study (17); there was no reported requirement for duration of pre-trial stability of levothyroxine dosage in one study of surgically-treated patients who had Graves disease (16). The study treatment periods ranged from 5 to 15 weeks (14–20). The details of combination therapy are shown in **Table 1**; only one study (14) reported twice daily administration of L-T3. A summary of the risk of bias of included trials is shown in **Table 2**.

In a random effects meta-analysis, the pooled prevalence rate for preference of combination therapy over L-T4 was 46.2% (95% CI 40.2%, 52.4%) ($p = 0.231$ for the difference from chance, using data from 7 trials including 348 hypothyroid individuals) (**Figure 2**). There was no significant statistical heterogeneity among study results ($Q = 7.32$, degrees of freedom [df] = 6, $p = 0.293$, $I^2 = 18.0\%$). A funnel plot investigating for publication bias was not performed due to an insufficient number of

TABLE 1 | Study and participant characteristics in the included randomized controlled trials.

References	Country	Trial design	Number randomized participant (Number reporting treatment preference outcome)*	Females	Mean age (Standard deviation) (years)	Etiology hypothyroidism	Description of combination L-T3* and L-T4† therapy	Study funding and industry participation
Appelhof et al. (14)	Netherlands	Parallel	141 (92 L-T3 arm, 48 L-T4 arm)	85% (120/141)	46.8–49.8 (9.4–9.8)	Autoimmune primary hypothyroidism (141/141, 100%)	Twice daily dosing. In respective study arms, 25 ug L-T4 removed and added L-T3, aiming for L-T4:L-T3 ratio of 5:1 or 10:1. For patient on 100 mcg LT4—for 5:1 becomes 75 ug L-T4 and 15 ug L-T3, for 10:1 becomes 75 ug L-T4:7.5 ug L-T3.	Academic institutional funding, Study medication provided by Merck, Netherlands
Bunevicius et al. (15)	Lithuania	Cross-over	35 (33)	94% (31/33)	46 (13)	Autoimmune primary hypothyroidism (16/33, 48%), thyroid cancer (17/33, 52%)	Once daily dosing. 12.5 ug L-T3 substituted for 50 ug of the usual L-T4 dose. For patient on 100 ug L-T4 becomes 50 ug L-T4 and 12.5 ug L-T3 (inferred 4:1).	Funding not reported. Study medication provided by Berlin-Chemie.
Bunevicius, et al. (16)	Lithuania	Cross-over	13 (10)	100% (13)	34 (NR‡)	Surgically-treated Graves disease (13/13, 100%)	Once daily dosing. 10 ug L-T3 substituted for 50 ug of the usual L-T4 dose. For patient on 100 ug L-T4, becomes 50 ug L-T4 and 10 ug L-T3 (inferred 5:1).	Not reported.
Escobar-Morreale et al. (17)	Spain	Cross-over	28 (26)	100% (28/28)	48 (11)	Autoimmune primary hypothyroidism (23/28, 82%); RAI-treatedμ Graves disease or Toxic Multinodular Goiter (5/28, 18%)	Once daily dosing. 5 ug L-T3 substituted for 25 ug of the usual L-T4 dose (all patients had baseline pre-trial L-T4 dose of 100 ug, so calculate 75 ug L-T4 and 5 ug L-T3, inferred ratio 15:1).	Academic and industry (Merck KgaA) funding.
Nygaard et al. (18)	Denmark	Cross-over	68 (59)	93% (55/59)	46.5—47.6 (12.3—13.1)	Autoimmune primary hypothyroidism (68/68, 100%)	Once daily dosing. 20 ug of L-T3 substituted for 50 ug of the usual L-T4 dose. For patient on 100 ug L-T4, becomes 50 ug L-T4 and 20 ug L-T3 (inferred 2.5:1).	Academic foundation funding.
Rodriguez et al. (19)	United States	Cross-over	30 (27)	83% (25/30)	47.5 (12.9)	Autoimmune primary hypothyroidism (23/30, 77%), Thyroidectomy (3/10, 10%), RAI-treated (4/30, 13%)	Frequency of daily dosing not reported (assume once a day). Aim for 5:1 ratio of L-T4:L-T3. 10 ug L-T3 substituted for 50 ug of the usual L-T4 dose. For patient on 100 ug L-T4, becomes 50 ug L-T4 and 10 ug L-T3.	Academic funding from the National Institutes of Health. Medication provided by King Pharmaceuticals.
Walsh et al. (20)	Australia	Cross-over	110 (101)	92% (101/110)	47.7 (11.7)	Autoimmune primary hypothyroidism (94/110, 85%) thyroidectomy for non-malignant reason (12/110, 11%), RAI (4/110, 4%).	Once daily dosing. 10 ug L-T3 substituted for 50 ug of the usual L-T4 dose. For patient on 100 ug L-T4, becomes 50 ug L-T4 and 10 ug L-T3 (inferred 5:1).	Academic institutional funding, L-T3 donated by Boots, Australia

L-T3, levo-triiodothyronine.

† *L-T4, levothyroxine.*

‡ *NR, not reported.*

μ *RAI-treated, radioactive iodine-treated.*

TABLE 2 | Quality assessment of the included randomized controlled trials.

Reference	Selection Bias (Randomization and allocation concealment)	Risk of bias due to deviation from intended interventions (e.g., adherence)	Risk of bias due to missing outcome data	Risk of bias in measurement of levo-triiodothyronine (L-T3) preference outcome with explanation	Selective outcome reporting
Appelhof et al. (14)	Low	Some concerns*	Low	Some concerns$^{\mu}$ Explanation: Subjective appreciation of L-T3 compared to pre-trial L-T4 was rated by the participants on a 5-point scale (much better, somewhat better, the same, somewhat worse, or much worse), and those who indicated much or somewhat better were categorized as preferring L-T3. Unclear if validated scale.	Low
Bunevicius et al. (15)	Some concerns*	Some concerns*	Low	Some concerns$^{\mu}$ Explanation: At the end of the trial, participants asked which treatment was preferred. Unclear if standardized instrument or wording or if what response options may have been provided.	Low
Bunevicius et al. (16)	Some concerns*	Some concerns*	High (23% loss randomized participants)	Some concerns$^{\mu}$ Explanation: At the end of the trial, participants asked which treatment was preferred. Unclear if standardized instrument or wording or if what response options may have been provided.	Low
Escobar-Morreale et al. (17)	Low	Low	Low	Some concerns$^{\mu}$ Explanation: At the end of the trial, participants asked which treatment was preferred. Unclear if standardized instrument or wording or if what response options may have been provided.	Low
Nygaard et al. (18)	Some concerns†	Some concerns*	Some concerns (13% loss randomized participants)	Some concerns$^{\mu}$ Explanation: At the end of the trial, participants asked which treatment was preferred. Unclear if standardized instrument or wording or if what response options may have been provided.	Low
Rodriguez et al. (19)	Low	Some concerns*	Some concerns (10% loss randomized participants)	Some concerns$^{\mu}$ Explanation: At the end of the trial, participants asked which treatment was preferred. Unclear if standardized instrument or wording or if what response options may have been provided.	Low
Walsh et al. (20)	Some concerns‡	Low	Low	Some concerns$^{\mu}$ Explanation: At the end of the trial, participants asked which treatment was preferred. Unclear if standardized instrument or wording or if what response options may have been provided.	Low

*****Insufficient detail reported in the manuscript.*

† *The levothyroxine component of combination therapy was open label for dose adjustment.*

‡ *Sealed envelopes were used but there was no report of whether these were opaque (to ensure that the treatment allocation was not visible through the envelope).*

$^{\mu}$ *Some concerns, if there was no validated questionnaire outcome measure for treatment preference.*

studies for meaningful interpretation (i.e., fewer than 10 trials in the meta-analysis).

In spite of the lack of statistically significant heterogeneity in our primary meta-analysis, we proceeded with planned sensitivity analyses, to explore for any potential differences in treatment benefits according to patient, study, and disease characteristics. We were not able to examine the impact of study duration as all of the included trials (14–20) were $\leq$ 3 months in duration; furthermore, we were not able to examine any potential impact of age, as the mean age of study participants was relatively young (<50 years of age) in all trials (14–20). In terms of study quality, there was no significant difference in combination therapy preference between 4 studies in which there were some concerns about selection bias (randomization/concealment of allocation) (15, 16, 18, 20) compared to 3 studies which were considered at low risk of bias for that variable (14, 17, 19) (between study heterogeneity $Q = 0.027$, $df = 1$, $p = 0.871$). There was also no significant difference between 5 studies that included some men (14, 15, 18–20), compared to two that included only women (16, 17) ($Q = 0.196$, $df = 1$, $p = 0.658$). Furthermore, there were no significant difference between 5 trials that included individuals who had a thyroidectomy or radioactive iodine treatment (15–17, 19, 20) compared to 2 trials that included only individuals with autoimmune primary

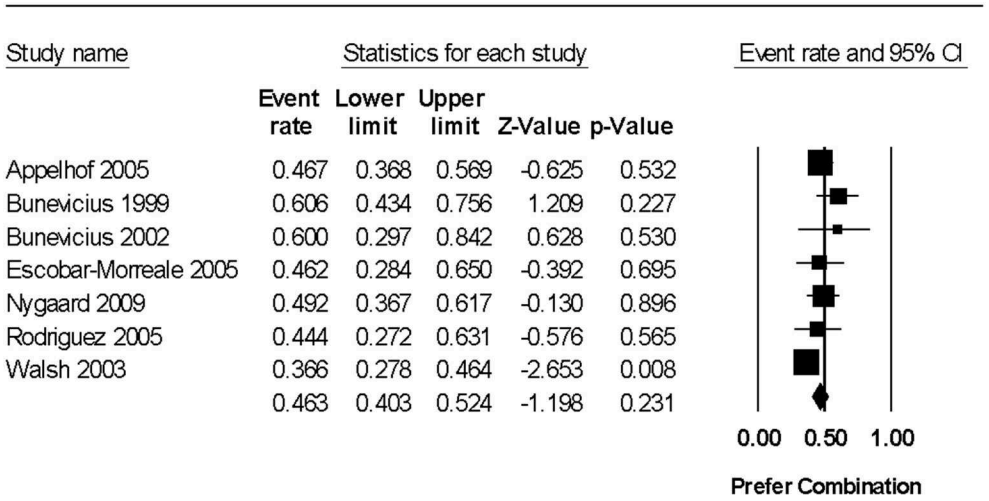

FIGURE 2 | Forest plot from a random effects meta-analysis examining prevalence of preference of combination levo-triodothyronine (L-T3) and levothyroxine (L-T4) therapy over L-T4 alone. 95% CI, 95% confidence interval. For the studies reporting the number of individuals who had no preference (and thus were assumed to favor standard care), the rates were as follows: Bunevicius et al. (15)−33.3% (11/33), Bunevicius et al. (16)−20.0% (2/10), Nygaard et al. (18)−35.6% (21/59), Rodriguez et al. (19)−29.6% (8/27), and Walsh et al. (20)−17.8% (18/101). It is not known if the individuals with no preference were indifferent or indecisive (i.e., unable to make a decision).

hypothyroidism (14, 18) ($Q = 0.083$, $df = 1$, $p = 0.773$). In examining the effect of frequency of dosing of combination therapy, there was no significant difference between one study that utilized twice daily dosing (14) compared to the 6 other studies that utilized once daily dosing or did not report on dosing (assuming once daily dose) (15–20) ($Q = 0$, $df = 1$, $p = 0.998$). In examining whether trials with end of trial TSH differences between treatment groups were associated with differences in treatment preferences, a trend for a possible marginal association was observed. Specifically the preference rate for combination therapy was 38.6% (95% CI 30.5%, 47.4%) in the 2 studies where the TSH was significantly higher in the combination therapy group compared to the L-T4 group (17, 20), 46.7% (95% CI 36.8%, 56.9%) in one trial where TSH was reduced in the combination therapy group compared to the L-T4 group (14) and 51.9% (95% CI 43.2%, 60.4%) in the 4 trials where end of trial TSH was not significantly different between treatment groups (15, 16, 18, 19) (between group difference for categories of TSH differences, $Q = 4.504$, $df = 2$, $p = 0.105$). In summary, study quality (reflected by randomization/concealment of allocation method), inclusion of males, inclusion of individuals who had a thyroidectomy or radioactive iodine treatment, and frequency of combination therapy daily dosing, did not explain combination therapy preference; however a possible marginal relationship between end of study TSH differences and treatment preference was observed.

In order to investigate any relationship between dose and treatment preference over L-T4 monotherapy taken during or before the trial, the respective L-T3 and L-T4 total daily dosage, and the ratio of these two doses, was calculated for a hypothetical baseline L-T4 dosage of 100 ug/day, according to each respective trial protocol (**Table 2**). Respective fixed effects meta-regression analyses were performed (**Figure 3**). Data from the L-T4 monotherapy arm in the parallel design randomized trial of Appelhof et al. (14) was included in these analyses, assuming that the L-T4 baseline dosage was continued and the L-T3 dosage was 0 ug/day. Data from 7 trials of 396 participants [incorporating 3 subgroups from the trial by Appelhof et al. (14)] were used in the meta-regression analyses. There was a statistically significant positive association between total daily L-T3 dosage (which ranged from 0 to 20 ug/day) and treatment preference (slope regression model 0.043, 95% CI 0.007, 0.078, $p = 0.020$) (**Figure 3A**). However, there was no significant association of treatment preference with the L-T4:L-T3 ratio of 1:0 to 4:1) (slope 0.124, −0.489, 0.738, $p = 0.691$) (**Figure 3B**).

We performed several sensitivity analyses of combination therapy preferences, where we grouped trials according to changes in other specific outcomes. Specifically, grouping studies that demonstrated differences between treatment groups for validated measures of quality of life, changes in body weight, mood, and symptoms. We found no significant difference in rate of preference for combination therapy in comparing one trial reporting improved quality of life with combination therapy (18) to 3 trials where there was no significant treatment group difference in any quality of life measure (14, 17, 20) ($Q = 1.644$, $df = 2$, $p = 0.439$). Furthermore, there was no significant difference in treatment preference rate in comparing one study in which body weight was statistically significantly reduced in the combination therapy group (14) to 5 other trials reporting no significant body weight difference ($Q = 0.637$, $df = 1$, $p = 0.723$). However, a marginally higher rate of preference for combination therapy (53.2%, 95% CI 42.9%, 63.2%) was observed in two trials reporting significant improvement in mood and symptoms (respectively) with combination treatment (15, 18),

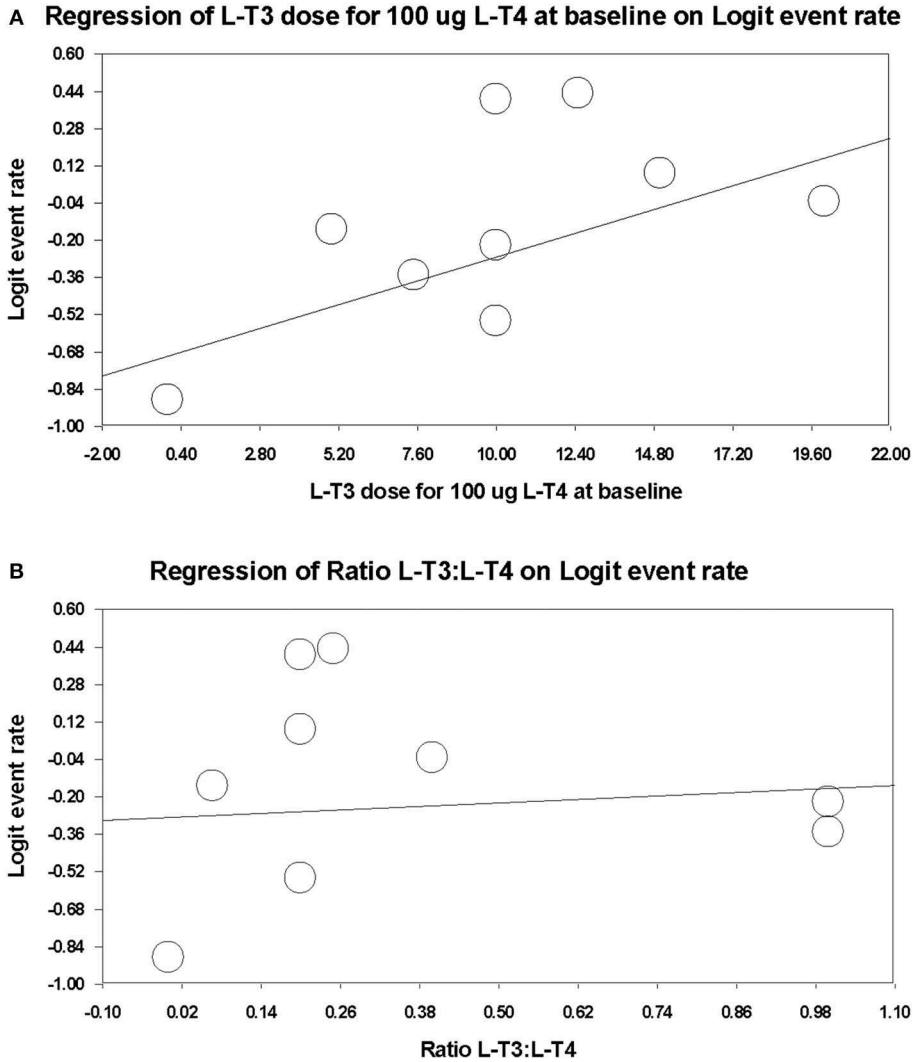

FIGURE 3 | Meta-regression plots examining for any dose-response relationship between combination therapy dose and treatment preference over L-T4 monotherapy (L-T4 monotherapy during or before trial). **(A)** Total daily L-T3 dosage (ug) on combination therapy. **(B)** Ratio of L-T3 divided by L-T4 dosage (ug) on combination therapy.

compared to 5 other trials where there was no significant difference between treatment groups in either measure (43.1%, 95% CI 37.1%, 49.2%) (14, 16–20) (between group difference $Q = 2.762$, $df = 1$, $p = 0.097$). In summary, the minority of trials reporting improvement in mood and symptoms, tended to report higher rates of combination therapy preference.

Although none of the primary reports of the trials in this systematic review included molecular biomarker data, given the importance of potential relationship between molecular characteristics of patients and treatment preference, reports of secondary publications from included trials were descriptively summarized. The methodologic quality of respective secondary analysis papers (21–23) was considered consistent with that of the original trials, so is not reported separately. In secondary analyses of original randomized trial data (14), Appelhof et al. compared rates of preference for combination therapy, according to genetic polymorphism status of type 2 deiodinase enzymes for Thr92Ala and ORFa-Gly3Asp (also known as rs12885300) (21). The prevalence rate of the Thr92Ala polymorphism among 141 trial participants was as follows: 74 (52%) heterozygous, 20 (14%) homozygous, and 47 (33%) wild type (21). The number of individuals and percentage with the ORFa-Gly3Asp polymorphism was: 52 (37%) heterozygous, 19 (13%) homozygous, and 70 (50%) wild type (21). Among the 92 patients who received combination therapy, no significant differences in rates of combination therapy preference were observed according to Thr92Ala polymorphism genotype (53% heterozygous, 39% homozygous, 41% wild type) nor ORFa-Gly3Asp genotype (49% heterozygous, 43% homozygous, and 46% wild type) (21). The authors concluded there was no

association between D2 polymorphisms and well-being or subjective preference for combination treatment over L-T4 monotherapy (21). In another secondary analysis of the same original trial by Appelhof et al. (14), Van der Deure et al. (22) examined polymorphisms in OATP1C1 gene, encoding a protein capable of thyroid hormone transport into the brain. Genotyping was successfully executed in140/141 patients in the original trial (22). The prevalence of OATP1C1-intron3C>T, OATP1C1-Thr143 and OATP1C1-3035T alleles were 46, 3, and 43%, respectively (22). Among 92 trial patients who received combination therapy, there was no significant difference in combination therapy preference according to genotype status: nOATP1C1-intron 3C ($p = 0.68$); OATP1C1-pro143Thr ($p = 0.22$), or OATP1C1-C3035T ($p = 0.95$ for respective chi-squared tests). However, in a secondary analysis of the trial from Nygaard et al. (18), Carlé et al. reported that in a subgroup of 45 patients from the 59 participants completing the original trial (18), the presence of the combination of 2 polymorphisms (rs225014 encoding the DIO2 enzyme and rs12885300 encoding the MCT10 transporter) was associated with a higher rate of preference for combination treatment: 63% if one polymorphism present, 100% if both polymorphisms present, and 42% if wild type ($p = 0.009$) (23).

DISCUSSION

In conclusion, in this systematic review and meta-analysis of relatively short-term blinded RCTs, approximately 46% of adult hypothyroid patients preferred combination therapy with L-T3 and L-T4 and L-T3 over L-T4 monotherapy; yet these findings were not distinguishable from chance. Some differences between this study with prior guideline narrative summaries of combination therapy preference rates (1, 32), is our strict inclusion criteria relating to blinding (to minimize the risk of bias), exclusion data from any add-on non-randomized treatment arms (also to minimize the risk of bias), and the a priori definition of statistical significance relative to chance in our meta-analysis. The fundamental clinical assumption of our analyses was that patients either prefer combination therapy to the standard of care or not, so patients who prefer L-T4 monotherapy or those who have no preference, would be grouped together as they would be treated with the same standard of care of L-T4 monotherapy. We found no patient demographic, disease, or study characteristics associated with variability in combination therapy preference. Studies reporting improvements in symptoms and mood (15, 18), tended to report higher rates of preference rates for combination therapy. The types of physical and emotional symptoms that were reported on questionnaires to be improved in these studies (15, 18) included: feeling cold (15), blurred vision (15), nausea (15), fatigue (15), depression/sadness (15, 18), anger (15), confusion (15), fearfulness (15), irritability (15), anxiety (18), and general health (18). However, the majority of studies did not report any significant difference in quality of life (using validated quality of life questionnaires) nor body weight with combination therapy and patient preference did not vary with these measures. Significant weight loss was reported only in a high dose combination therapy arm (5:1 L-T4 to L-T3 dose ratio) in one trial, where the TSH was suppressed with combination therapy (14). There was some preference variability of marginal statistical significance associated with end of trial TSH difference between study groups; specifically, for trials reporting an end of trial combination therapy group TSH that was either significantly lower or higher than the L-T4 monotherapy arm, tended to be associated with diminished patient preference for combination therapy. A positive association of L-T3 total daily dosage and treatment preference was observed in an exploratory univariate meta-regression analysis, where the dosages of L-T3 varied from zero to 20 ug/day. We were not able to make any firm conclusion on any potential relationship between patient molecular characteristics and combination therapy due to paucity of data. The secondary analyses summarized in this review should be interpreted as hypothesis generating and further confirmatory research is needed.

It is important to acknowledge that in the clinical practice setting, patient preference rates for combination therapy may differ from those observed in blinded randomized trials, particularly if patients may have some negative pre-established perceptions of L-T4 therapy (nocebo effect) and positive expectations with combination therapy (particularly L-T3). In clinical practice, experiences of patients treated with combination therapy may be highly variable, including the reasons preferring combination therapy (or not), and the degree of benefits on symptoms, well-being, and functional ability. However, the potential therapeutic benefits are likely be enhanced in a supportive, encouraging clinical care environment where patients' symptoms and concerns are acknowledged and their views incorporated in medical decision-making. Of note, intense monitoring for treatment benefits (e.g., using detailed questionnaires) and adverse effects is expected to be more rigorous in a research trial setting compared to clinical practice. The experience of clinicians prescribing and adjusting the dose of combination therapy may be also different in clinical practice compared to the clinical trial setting (e.g., specialized clinical trial centers with investigators experienced in use of combination therapy). In an effort to address physician expertise, authors from the European Thyroid Association have recommended that only specialists accredited in Endocrinology or Internal Medicine should be the ones prescribing combination therapy (32). Yet even among endocrinologists, experience prescribing combination therapy may be variable. Some practical potential challenges in clinically utilizing combination therapy using commercially-available existing short-acting L-T3 preparations may include: complexity of administration of two different thyroid hormone preparations (often more than once a day for higher total daily doses), increased medication expense (the extent of which varies globally), and greater complexity/expense in medication monitoring (e.g., inclusion of T3 measurements in bloodwork, potentially including peak levels). Saravanan et al. have reported that in hypothyroid patients receiving combination therapy, where the baseline L-T4 dose is reduced by 50 ug and replaced with 10 ug of L-T3, peak blood free T3 levels rise 42%

(4 h after dose administration) (33). As such, use of higher dose L-T3 in combination treatment may necessitate measurement of peak T3 blood levels and splitting of the doses of this hormone. The extent to which such levels are faithfully reflected by diverse tissue requirements further confounds optimal dosing of L-T4/L-T3. Authors from the European Thyroid Association have also suggested a relatively physiologic combination therapy dose ratio L-T4/L-T3 ranging from 13:1 to 20:1 by weight (administering L-T4 once daily and dividing the total daily L-T3 dose in two doses) (32). The ETA guidelines regarding combination therapy were developed to enhance safety (potentially due to harms from treatment with supraphysiologic doses of thyroid hormones) and to counter its indiscriminate use (32). The ETA guidelines have indicated that "the goal of L-T4/L-T3 combination therapy is to resolve persistent complaints despite a normal TSH in L-T4-treated hypothyroid patients" (32). Furthermore, in the interest of safety, the ETA guidelines have recommended close specialist follow-up, with dose adjustments intended to meet the goals of treatment (32). Authors from the Italian Society of Endocrinology and the Italian Thyroid Association have suggested a dose ratio of L-T4:L-T3 of 10 to 20:1, administered in divided daily doses (3). However, the suggested relatively physiologic L-T3 dosages are below that used in many of the trials included in this review. One of the potential risks of using higher dose L-T3 may be TSH suppression, particularly if the L-T4 dosage is not sufficiently reduced, and TSH suppression with combination therapy was reported in some of the included trials. The Italian guidelines also highlight the importance of close monitoring for potential adverse effects, including cardiovascular complications and osteoporosis (3). Relative contraindications to L-T3 combination therapy are important considerations. Clinical practice guideline authors sponsored various organizations have recommended avoiding combination L-T3/L-T4 therapy in the following groups: pregnant women (3, 6, 32, 34) the elderly (3) patients with known cardiac arrhythmias (6, 32), individuals with cardiac risk factors (3), patients with differentiated thyroid cancer with a high risk of disease progression or intermediate to high risk of adverse effects (3).

There are multiple strengths and several limitations of this systematic review and meta-analysis. An important strength is the systematic search for relevant citations conducted in multiple electronic databases by an experienced library information specialist (which was supplemented by a hand search). Furthermore, two reviewers independently review of citations and full-text papers in duplicate, with resolution of any discrepancies in inclusion of papers resolved in discussion with a third content expert reviewer. Two investigators also independently critically appraised included studies and abstracted the data, with the final consensus of reported results. We also contacted some authors of primary studies to obtain critically information, relating to study inclusion and results. Some limitations of this research include: exclusion of non-English studies (due to lack of resources for translation), lack of a comprehensive search of the gray literature, inclusion of a relatively small number of trials (such that publication bias could not be reliably assessed), some methodologic limitations of included trials, and short duration of included trials (precluding analysis of durability of patient preference over time). Additional potential limitations relating to treatment of hypothyroidism that were not addressed by this review nor the included studies include the potential for a symptom-optimized TSH goal that may be narrower than the traditional 95% reference range (35), drug interference with TSH secretion (36), management of treatment-refractory hypothyroidism (i.e., individuals requiring unusually high doses of thyroid hormones) (37), and consideration of interference with gastrointestinal absorption of thyroid hormones (38).

In conclusion, although L-T4 monotherapy is the standard of care in management of hypothyroidism in adults, dissatisfaction among some patients treated with L-T4 as well as significant uncertainties relating to thyroid hormone alternatives, highlights the critical need for more research on effective treatments to optimize the well-being and treatment satisfaction in this population.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

All of the authors provided input on the design of the study and reviewed the manuscript. RF conducted the electronic database search. AA and AS screened citations, reviewed the full-text papers, critically appraised included studies, abstracted the data, and drafted the manuscript. SE provided input, in the event consensus was not achieved by AA and AS on inclusion of studies. AS conducted the statistical analyses and the statistical methods and data were reviewed by LT.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2019.00477/full#supplementary-material

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The remaining 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.

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Sustained Release T3 Therapy: Animal Models and Translational Applications

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The standard of care to treat hypothyroidism is daily administration of levo-thyroxine (LT4). This is based on the understanding that deiodinases can restore production of T3 and compensate for the small amounts of T3 that are normally produced by the thyroid gland. However, pre-clinical and clinical evidence indicating that deiodinases fall short of restoring T3 production is accumulating, opening the possibility that liothyronine (LT3) might have a role in the treatment of some hypothyroid patients. LT3 tablets taken orally result in a substantial peak of circulating T3 that is dissipated during the next several hours, which is markedly distinct from the relative stability of T3 levels in normal individuals. Thus, the effort to developing new delivery strategies for LT3, including slow release tablets, liquid formulations, use of T3-related/hybrid molecules such as T3 sulfate, poly-zinc-T3 and glucagon-T3, nanoparticles containing T3, subcutaneous implant of T3-containing matrices, and stem cells for de novo development of the thyroid gland. This article reviews these strategies, their applicability in animal models and translatability to humans.

Keywords: hypothyroidism, liothyronine, combination therapy, animals, thyroid, levothyroxine

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INTRODUCTION

Hypothyroidism is a common disorder that in most cases results from insufficient activity of the thyroid gland (1). The thyroid secretion contains both thyroxine (T4) and triiodothyronine (T3), with the latter being the active form of thyroid hormone (TH). It is estimated that healthy adult subjects produce approximately 100 ug T4 daily; the daily production rate of T3 is approximately 30 ug, of which only about 5 ug are secreted from the thyroid, with the remaining 25 ug produced via T4 deiodination outside of the thyroid parenchyma (2). During hypothyroidism, the reduction in circulating TH levels is detected by the hypothalamus-pituitary-thyroid (HPT), hence elevating serum TSH and defining the diagnosis of hypothyroidism (3). From the time it was originally described in the late 18th century until the 1970s, hypothyroidism was treated predominantly with desiccated thyroid extract, which contains both T4 and T3 (4). The discovery that humans convert T4 to T3 and the development of a TSH immunoassay that could be used to titrate the thyroid replacement dose, levothyroxine (LT4), became the standard of care and the need for supplementation with LT3 obviated; more recently combined therapy with LT4 and LT3 has been the subject of much discussion and controversy (4).

The thyroid function is activated by the pituitary hormone TSH, which is under direct hypothalamic stimulation via TRH and inhibition via a feed-back negative mediated by circulating T4 and T3, each playing independent roles (5). Plasma T3 is detected directly by the hypothalamic

paraventricular nucleus, where TRH is produced, and in the pituitary thyrotrophes, where TSH is secreted. In contrast, in order to slow down expression of TRH and TSH, plasma T4 requires local conversion to T3 via the type 2 deiodinase (D2), which is present in the hypothalamus and in the anterior pituitary gland (6). The independent role of T4 is observed as serum TSH increases with the drop in serum T4 during iodine deficiency or mild hypothyroidism, while circulating T3 remains within normal range (7, 8). In turn, the acute administration of large doses of PTU to thyroidectomized individuals kept on L-T4-replacement therapy revealed how serum T3 *per se* has an important role in TSH secretion (9). The approximately 20% drop in serum T3 that follows as a result of D1 inhibition is sufficient to double serum TSH levels, even as serum T4 levels remain stable (9).

TH signaling is initiated via binding of T3 to nuclear receptors (TR) (10). Based on the affinity of TRs for T3, it is considered that normal circulating levels of T3 account for the bulk of TH signaling in target tissues (11). In some organs, however, the activity of local deiodinases can modulate TH signaling provided by plasma T3. Tissues that express D2 such as brain, pituitary gland and brown adipose tissue, have enhanced TH signaling because D2 produces T3 locally, which adds to the incoming T3 from circulation. In contrast, TH signaling is dampened in tissues expressing the type 3 deiodinase (D3), such as placenta, pancreatic beta cells, skin, which inactivates both T4 and T3 (11). Given that tissues and plasma T3 are in equilibrium, deiodinases produce most circulating T3 and play a role in maintaining circulating levels of T3 relatively stable. Notwithstanding, the thyroid gland also sustains plasma T3 levels as seen in animal models of deiodinase deficiency (12, 13). In fact, TSH acts on the thyroid by preferentially accelerating T3 secretion (over T4) (14–16). For example, the minimal circadian rhythmicity observed in plasma T3 levels is thought to result from an elevation in circulating TSH in the early morning hours [see (11) for review].

The standard of care for hypothyroidism is treatment with levothyroxine (LT4) that is adjusted based on serum TSH levels. The goal is to give patients sufficient amounts of LT4 to bring serum TSH within the normal reference range (3). However, since the early 70s it became apparent that therapy with LT4 results in significantly higher T4 and lower T3 serum levels, what was attributed to the absence of thyroidal T3 secretion (17). Nevertheless, a later study from the same group reported that LT4-treated patients have normal T3 and significantly higher T4 serum levels (18). In addition, a non-cross sectional study that looked at serum T3 levels in 50 euthyroid individuals before and after thyroid surgery found that therapy with LT4 can restore both circulating TSH and T3 levels (19). Hence the expectation that in LT4-treated patients the deiodinase system will metabolize LT4 and produce T3 in amounts equivalent to what is produced by the healthy thyroid gland. However, there is evidence indicating that this indeed might not be the case (20, 21). In a series of approximately 2,000 LT4-treated hypothyroid patients, serum T3 is relatively lower and serum T4 is relatively higher when compared to control individuals; in about 15% of the patients, serum T3 is below normal range (22). Similar findings were obtained through the analyzes of publically available NHANES data, showing that approximately 500 individuals maintained on LT4 have lower serum T3 levels when compared with control individuals matched for age, sex, ethnic background and serum TSH (23).

Preclinical studies of LT4-treated thyroidectomized rats indicate that minimally reduced plasma T3 levels are sufficient to cause widespread dampening in TH signaling, including in the liver, skeletal muscle and brain (24). There is also evidence that TH signaling might not be fully normalized in LT4-treated hypothyroid patients. For example, in association studies LT4-treated patients that have a normal serum TSH weigh about 10 pounds more despite ingesting less calories; they report less physical activity and are more likely to be on statins and anti-depressive therapy (23). A meta-analysis of LT4-treated patients revealed that both total cholesterol and LDL cholesterol remains elevated despite serum TSH that was within the reference range (25). In fact, these findings go along with the observation that non-objective symptoms of hypothyroidism remain in about 15–20% of the LT4-treated hypothyroid patients (difficulty with weight management, low energy, depressed mood, and memory impairment) despite achieving normal TSH and free T4 levels (26, 27). However, progress in this area is slow given the multiple biases which impact on subject self-reported quality of life that are not thyroid related.

The use of liothyronine (LT3) in combination with LT4 to treat hypothyroidism is not frequent but it is popular among patients that, despite normal circulating TH and TSH, experience residual symptoms (28). However, available oral LT3 preparations result in rapid T3 absorption that is followed by fast metabolization (29). This typically results in a peak of serum T3 at about 3h after dosing, which is followed by a relatively fast decline in serum T3 levels (29–31) (**Figure 1**). In patients taking 10 ug of LT3, the serum T3 peak is approximately 40% above the baseline levels (32); multiple daily doses might partially mitigate this problem but its practicality is a challenge. The peak of serum T3 observed after oral administration of LT3 is not physiological and has given pause to physicians that wish to prescribe LT3. Thus, the American Thyroid Association recommends "against the routine use of combination treatment with LT4 and LT3 as a form of thyroid replacement therapy in patients with primary hypothyroidism" (3). Subsequently, in an attempt to identify adverse outcomes for patients on LT3 tablets, cardiovascular, skeleton, and mental outcomes were assessed through an observational study in the Scottish town of Tayside (33). Compared to the about 34,000 patients taking only LT4, those using LT4 plus LT3 ($n = 327$) or LT3 alone ($n = 73$) had no increased mortality or morbidity risk due to cardiovascular disease, atrial fibrillation, or fractures after adjusting for age; the number of prescriptions for bisphosphonates or statins were also similar. A novel finding was an increased risk of new

Abbreviations: AUC, area under the curve; Cmax, maximum concentration; D2, type 2 deiodinase; D3, type 3 deiodinase; FT4, free thyroxine; FT3, free triiodothyronine; HR, (place in column) heart rate; HPT, hypothalamus-pituitary-thyroid; LT3, liothyronine; LT4, levothyroxine; mESC, mouse embryonic stem-cells; PZL, Poly-Zinc-Liothyronine; PK, Pharmacokinetics; T3S, T3 sulfate; T3, triiodothyronine; T4, thyroxine; Tmax, time to maximum concentration; TSH, thyroid stimulating hormone.

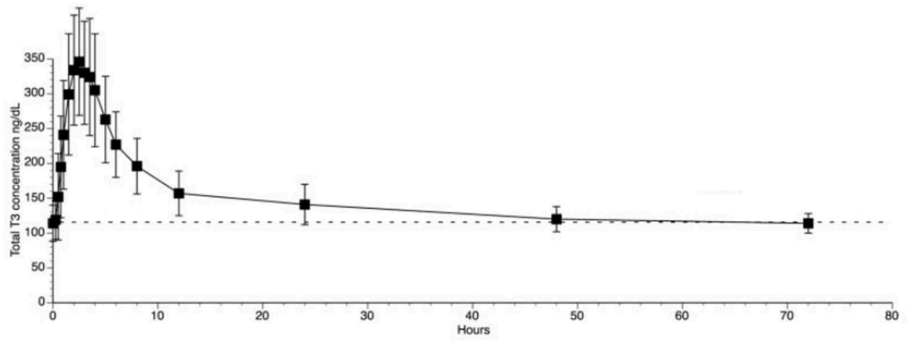

FIGURE 1 | T3 serum concentrations for 3 days following oral administration of a single 50 mcg dose of liothyronine to volunteers. Adapted from Jonklaas et al. (30).

prescriptions for antipsychotic medication, proportional to the number of LT3 prescriptions (33). However, prospective safety studies to evaluate potential long term undesirable cardiovascular and skeleton associated with such pharmacokinetics (PK) have not been performed (34).

Delivery Systems That Aim at Physiological Replacement of T3

The utilization of animal models to develop systems that restore physiological levels of T3 has two main purposes. First, to study thyroid economy and TH action in other species. This is justified because hypothyroidism not only affects about 5% of the human population but is also frequently observed in other animal species such as dogs and cats (35, 36). Second, to develop delivery systems that eventually could be transitioned/adapted to human use, which has been done mostly in small laboratory rodents such as rats and mice. When studying or using animal models, however, one should be aware that thyroid economy in various species might not emulate what happens in humans (37). For example, rodents secrete relatively more T3 from the thyroid gland (about 40% of the daily production rate) and exhibit an approximately 12–24 times faster turnover rate for both serum T4 and T3 (2). However, when focusing on the absorption kinetics, rodents exhibit a similar PK profile as humans, i.e., a serum peak at 3 h after dosing that is followed by a fast decline in its circulating levels (38).

Enteral/Gastrointestinal Preparations

Oral

Administration of LT3 through the mouth can be utilized to restore TH signaling in laboratory animals that have been made hypothyroid. Adding LT3 to the drinking water or to the diet avoids the administration of a single dose of LT3 and has been shown to restore thyroid status in thyroidectomized rodents (37). However, consistency of dosing and the inevitable circadian rhythmicity associated with food and water intake remain a limiting factor. For example, LT3 supplied in the drinking water (dilution from a stock solution prepared in 40 mM NaOH) was given to different mouse strains aged 6–8 weeks, with variable results. Some strains exhibited complete (>90%) suppression of serum T4 and TSH whereas in others, a much weaker effect was observed. In addition, female and male animals responded differently, and heavier mice had higher post mortem levels of serum T3, which might have been due to increased water intake and/or changes in volume of distribution (39, 40). Notably, liquid preparations for oral administration of LT3 are available for humans (IBSA, Switzerland). Some patients might find it practical to use LT3 solutions, but few studies have been performed to date (41). Only one systematic study exists with this formulation but unfortunately PK profiles were not obtained (41).

New platforms continue to be developed to improve dosing flexibility and PK properties for oral administration of LT3. An original concept is the utilization of thermal inkjet (TIJ) 2D printing to enable the deposit of LT3 and/or LT4 onto orodispersible films (ODFs) (42). In a recent study, a two-cartridge TIJ printer was used to print separate solutions of LT3 and LT4. Dose adjustments allowed for LT3 (15–50 μg) and LT4 dosages (60–180 μg) to be successfully printed onto ODF. When placed in water, ODFs disintegrated in less than 45s (42). PK studies have not been performed but the prospect of fine customization of LT4 and LT3 dosages is exciting. Furthermore, it is conceivable that the utilization of different ODFs could effectively modify the PK properties of orally administered LT3.

An alternative approach to improve LT3 PK properties when given orally is to delay its absorption. This has been used for LT3 or combinations of LT4+LT3. For example, "slow release" LT3 tablets containing a hydrophilic swellable matrix system made with hydroxypropylmethylcellulose, sodium carboxymethylcellulose, calcium phosphate and magnesium stearate have been prepared (US Patent #5,324,522). Other combinations of salt and matrices have also been tested, including mannitol, magnesium stearate, calcium phosphate, and microporous polypropylene (US Patent #5,324,522). When tested *in vitro* the rate of LT3 release from such capsules can be modulated according to their content and grade of Methocel, and/or SimpleCap/Lactose (43). *In vivo* tests were also performed. In one of the two clinical studies performed to date, slow release LT3 tablets were given to 17 hypothyroid individuals. The results indicated that slowing down the release of LT3 in the intestine decreases the peak T3 in the serum (Cmax) by about 9% and prolonged the time to Cmax from ∼3.2

to 5 h (44). However, in the other study in which LT3 tablets were prepared with microcrystalline cellulose and magnesium stearate (BCT303), sustained serum T3 levels were not observed (30). Another approach still under testing is to deliver LT3 via chewable ion exchange resin that form a drug resin complex (Spectrix, Southlake TX). This technology utilizes ion exchange resin to form a multi-particulate drug-resin complex that could potentially provide an enhanced drug release profile and PK profile in humans, but clinical studies have yet to be performed.

To meet the challenge of creating sustained and/or slow release delivery systems for LT3, investigators utilized T3 derivatives. For example, it is well-known that phenolic hydroxyl within the T3 molecule can be sulfated (T3-S), a reaction that inactivates T3 but dramatically enhances its solubility in water and loss to the environment. However, sulfatases in the liver can reactivate T3-S via de-sulfation and prevent its loss to the environment (45). Studies in hypothyroid rats revealed that substantial amounts of parentally administered T3-S were converted back to T3, triggering systemic thyromimetic effects (46). Similar studies were conducted in 28 thyroidectomized individuals given T3-S orally. Tmax for T3-S was between 3 and 4 h. At the same time, T3 levels (generated through T3-S de-sulfation) increased rapidly in the circulation, with an early peak between 2 and 4 h that was followed by a variable plateau that depended on the dose of T3-S administered and lasted up to 48 h. These observations suggest that sufficient amounts of orally administered T3-S are converted to T3 in humans and could be tested as a tool to restore T3 levels in hypothyroid individuals (47). However, studies in euthyroid individuals have not been conducted. This is important because T3-S is metabolized through the D1 pathway through inner ring deiodination, which leads to irreversible inactivation of the T3 molecule (45). In fact, D1 is expressed in such high levels in the liver and kidney that it has been proposed that D1 plays a scavenger role by minimizing the loss of iodine through urine and bile (48). This is critical given that D1 is a T3-responsive gene, hence its levels are low in tissues of hypothyroid patients. Studies could be conducted in hypothyroid individuals after thyroid status was normalized with LT4 to ensure that at the normal D1 levels there is sufficient amounts of T3-S to be de-sulfated to T3. Nevertheless, this remains an ingenious strategy that should be pursued further.

Another T3 derivative was prepared using metal coordination, which resulted in LT3 polymers with Zinc. Poly-Zinc-Liothyronine (PZL) is a copolymer of zinc and T3 that forms a supramolecular complex (**Figure 2**). These supramolecular complexes of the form $[M(T3)]_n$ have superior mucoadhesive properties, and when coupled with the hydrolysis behavior of $[M(T3)]_n$ complexes, translate into a slow release formulation of LT3 (38). The properties of a controlled release, orally delivered drug product via metal coordination of a drug ligand, relies on known principles of mucoadhesion and coordination chemistry (49). For illustrative purposes only, consider PZL as a prodrug. Then the process of modified drug release and absorption can be seen to involve three distinct steps: (a) Mucoadhesion of PZL to an area of the gastrointestinal tract, (b) Controlled ligand exchange (e.g., hydrolysis) of T3 from PZL, followed by c) Drug absorption of the LT3. Many metal complexes, including PZL, exhibit mucoadhesion due to the interaction of the metal, acting as a Lewis acid, with anionic components of the mucosa (**Figure 3**) (50). Mucoadhesion prolongs the residence time (i.e., delays transit time) of a drug in the gastrointestinal tract (51). PZL interacts with the mucosa by a variety of additional mechanisms, including coordinate covalent bonding, hydrogen bonding, halogen bonding, metal- halogen bonding, electrostatic interactions and particle size (52–55). Thus, PZL adheres to the intestinal lining creating a "drug depot" from which LT3 gradually releases into the intestinal lumen and is ultimately absorbed into the bloodstream (**Figure 4**). Intestinal contents, such as bile acids and pancreatic secretion, accelerate breaking of the bonds between the metal and T3. Once freed from the metal, the released T3 reverts to its original form and quickly travels through the epithelial cell layer and into the blood stream. These processes increase the length of time PZL remains in the intestine and slows down the rate at which LT3 becomes available for absorption. Of note, Zn is an essential mineral involved in multiple physiological functions and the amount contained in a 30 ug dose of PZL is $<$ 1/1,000 of the daily recommended allowance.

Capsules of PZL were tested in hypothyroid rats via oral gavage, with a significant modification in PK properties (38). After PZL administration serum T3 exhibited about 30% lower peak (lower Cmax) that was delayed (longer Tmax) by about 6h as compared to rats given equimolar amounts of LT3. These figures could be further improved by packaging PZL in slow release capsules made with hydrophilic swellable matrix as described above for LT3 (44); the T3 clearance rate did not show differences between PZL- and LT3-treated rats. TSH levels, which were elevated, declined rapidly after LT3 administration but in PZL-treated rats the decline was delayed by about 4 h (**Figure 5**). Daily administration of PZL for 8 days showed that PZL and LT3 had similar long-term biological effects such as reduction of serum cholesterol, restauration of growth rate and induction of T3-dependent genes in the heart, liver and brain (38).

Rectal

The walls of the rectum are lined with a mucosa that is highly vascularized, allowing for rapid absorption of medications. Formal PK studies after the administration of the LT4 suppository have been performed in thyroidectomized rats (56). Tmax is at about 15 h, similar to when LT4 is given orally, but the area under the curve is about 3 times smaller indicating incomplete absorption. Similar results were obtained in hypothyroid patients (56). Unfortunately, no such studies have been performed with LT3 but should be explored.

Parenteral Preparations

Subcutaneous

One of the most common forms of TH replacement in small rodents is the subcutaneous injection of aqueous solutions of LT3. While this is a very reliable way of delivering exact amounts of LT3, its absorption is relatively fast, followed by a peak of LT3 in the circulation. Peaks can be minimized by splitting the dose into multiple injections every 24 h, but with a half-life of $\sim$2 h,

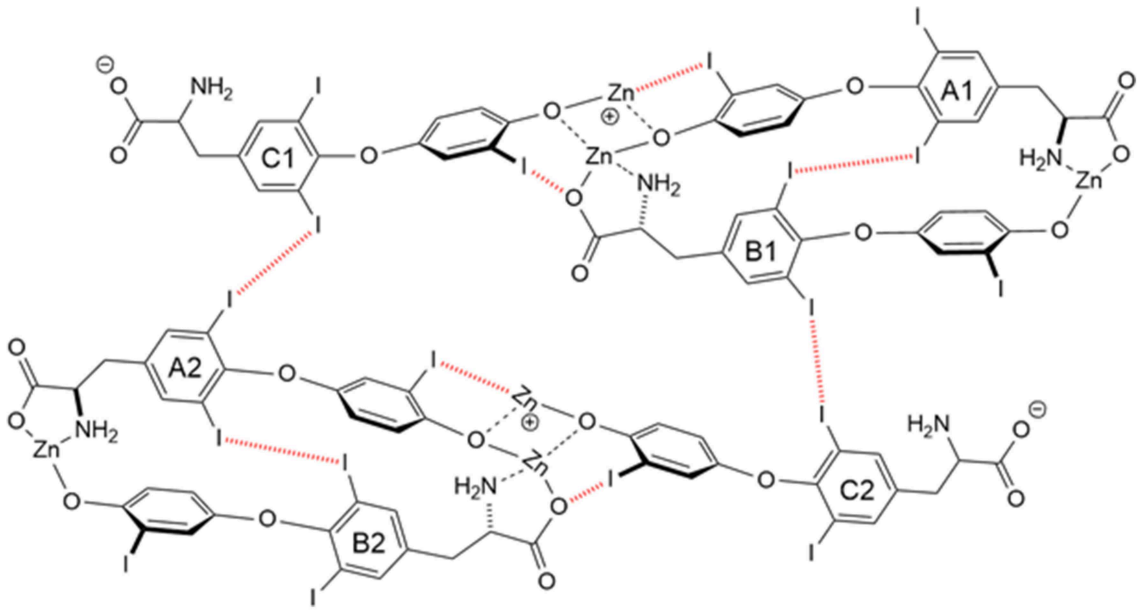

FIGURE 2 | Strong bonding interactions (coordinate covalent bonds) between Zn and ligand donor atoms of $T3^{2-}$ are shown in black; weak bonding interactions (halogen bonds) between iodine and X-bond acceptor atoms of $T3^{2-}$ are shown in red dashed line. Both bonding modes contribute to polymer formation and stabilization. Two and 3-D structures, including metal organic frameworks, are possible.

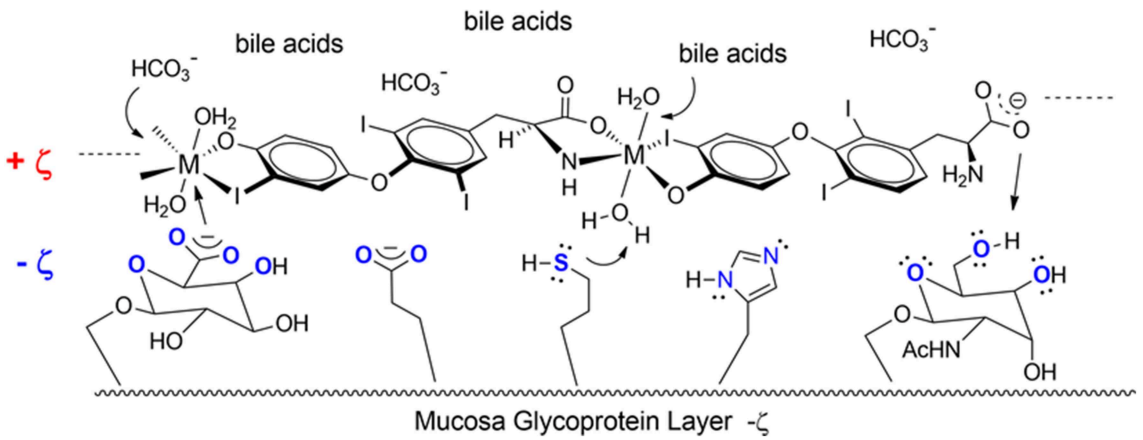

FIGURE 3 | Endogenous ligands in the upper GI tract can affect hydmlysis rate. These include HCl (stomach), bile acids and carbonate buffers (upper intestines).

the peaks of T3 in the circulation will inevitably remain (37). A more stable T3 profile could in theory be achieved by injecting an oil suspension of LT3, which should slow down its absorption rate, but formal PK studies remain to be done.

The gold standard in parenteral preparations for animals is the use of osmotic pumps or pellets that are implanted subcutaneously, releasing fixed amounts of T3 daily for a pre-defined number of days. The pump is a small cylindrical reservoir that uses osmotic driving agent to release a pharmaceutical of interest at a rate of 0.1 to 10 ul/h up to 6 weeks. Both the pumps and the pellets are suitable to be surgically implanted in rodents as well as in larger animals (37). Osmotic minipumps (Alzet, Cupertino CA) operate due to a difference in osmotic pressure between the pump and the subcutaneous area where the pump is implanted. The higher osmolality of the pump flux water through a semipermeable membrane into the pump. As the water builds up into the pump, it compresses the reservoir, displacing the pharmaceutical solution into the subcutaneous at a known rate (**Figure 6**). The pellet system (Innovative Research of America, Sarasota FL) consists of a pellet with a biodegradable matrix that continuously releases the pharmaceutical product in the animal. Pellets vary in diameter (1/8″ to 1/2″) depending on the pharmaceutical load and release rate. Pellets might contain from 0.001 mg up to 200 mg, which is released over a predefined period

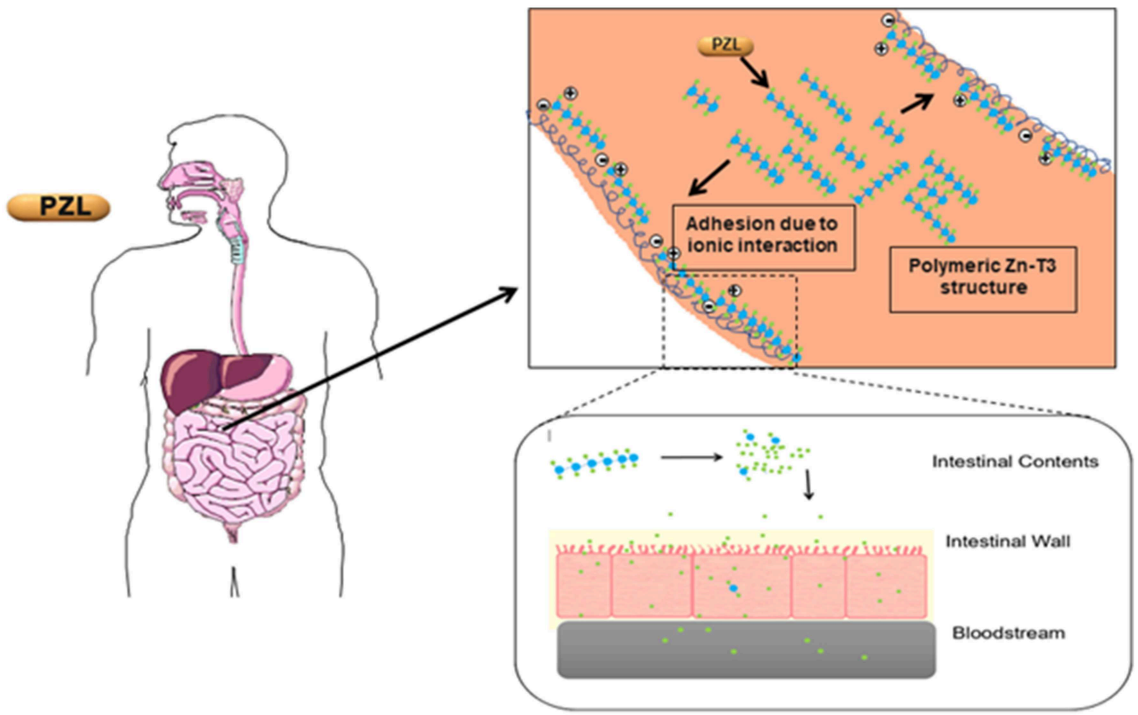

FIGURE 4 | Increasing the period PZL remains at the site of absorption and slowing the rate at which T3 becomes available for absorption, more closely matches T3 production in a healthy subject. By creating a unique "drug depot" from which T3 slowly releases, PZL addresses many of the limitations that have plagued previous attempts to mimic the natural production of T3 in healthy subjects.

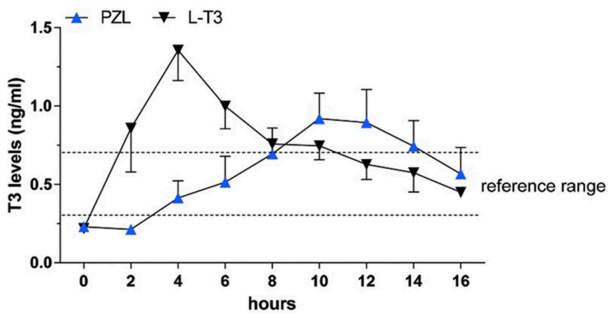

FIGURE 5 | Serum triiodothyronine (T3) levels in hypothyroid rats given 24 μg/kg body weight LT3 or equimolar amounts of PZL through gavage; *adapted fivm* Da Conceicao et al. (38).

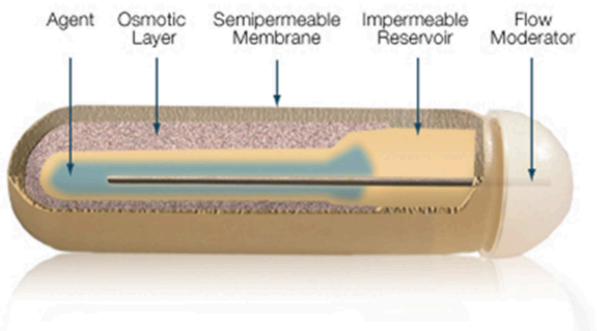

FIGURE 6 | Mechanism of ALZET osmotic pumps which relies on the osmotic pressure difference between pump's compartments. Reproduced from www.Alzet.com.

of time, e.g., 21 days, 60 days or 90 days. Thyroidectomized rodents implanted with either osmotic pumps or pellet devices do exhibit rather stable levels of T3 in the circulation (37).

Similar technology is being developed by pharmaceutical companies which could provide stable delivery of LT3 to patients and be applicable in the treatment of hypothyroidism. Titan Pharmaceuticals (South San Francisco CA) utilizes a proprietary technology (ProNeura®) to develop a platform to deliver LT3 subcutaneously. It consists of a solid rod made with a mixture of LT3 and ethylene-vinyl acetate that is placed subcutaneously, normally in the inner part of the upper arm, and can be removed at the end of the treatment period. Preliminary studies have been performed in thyroidectomized rats and in intact beagle dogs (57). LT3 implants continuously released T3 in both species, in a dose dependent manner for over 6 months. Following implantation, there was a transient serum peak of T3 followed by a sustained period of relatively stable circulating levels of T3 (57).

MedinCell (Montpellier, France) takes advantage of the fact that copolymers combined with pharmaceuticals are solubilized in a biocompatible solvent, which forms a fully bioresorbable

TABLE 1 | Developmental stage of different LT3 products, their main properties and PK studies in rodents and humans.

Delivery Route/Formulations	Properties of LT3 Preparations	
	Humans	**Rodents**
ENTERAL		
Na salt	- PK marked by fast absorption and a 3–4 h peak in serum that subsides after several hours (29)	- Similar PK to humans when given through gavage; mixed with food (38)
Slow release tablet	- Coated tablets; minimal/no PK change (30, 44) - Chewable LT3-resin complex gum; under development	- Not tested - Not tested
Liquid	- Commercially available (41); no PK studies	- Dissolved in drinking water; variable results depending on animal's weight and sex (37)
T3-Sulfate	- Some PK improvement with variable results (47)	- No PK studies; low potency effects (46)
PZL capsule	- Phase 1 clinical trial in 1 year	- Improved PK; similar thyromimetic effects (38)
PARENTERAL		
Subcutaneous		
Bioresorbable depot	- Under development; steady release for months	- Not tested
Ethylene-Vinyl Acetate Rod	- Not tested	- Relatively stable serum T3 levels for months (57)
Aqueous/oil solutions	- Not used	- Fast absorption with peak of T3 in the circulation
Osmotic pumps/pellets	- Not available in humans	- Stable levels of T3 in the circulation (37)
Intravenous	- Immediate peak of T3 in the circulation	- Immediate peak of T3 in the circulation (37)
Intraperitoneal	- Not used	- Rapid peak of T3 in the circulation (37)
REGENERATIVE METHODS		
Thyroid development from stem cells and transplant	- Not available	- Restored euthyroidism to hypothyroid nude mice; serum T3 levels presumably stable (58)
TISSUE-T3 TARGETING		
Hybrid molecules	- Not tested	- Glucagon-T3; no PK studies; liver-specific thyromimetic effects (60)
Nanotechnology	- Not tested	- Nanoparticles containing LT3; no PK studies; brain-specific thyromimetic effects (62)

depot once injected subcutaneously. BEPO® consists of copolymers containing hydrophilic blocks (polyethylene glycol—PEG) linked with hydrophobic blocks (Poly(D,L-lactic acid)—PLA), which precipitate and create a depot when placed an aqueous environment. The pharmaceutical is trapped within this matrix and later released by diffusion. The kinetics of pharmaceutical release can be fine-tuned by adjusting the hydrophilicity and relative ratio of the copolymers. Preliminary studies are not available but this technology could also potentially provide a steady release of LT3 molecules for days, weeks or months.

Intravenous and intraperitoneal

Both routes have been used to administer LT3 dissolved in aqueous solutions but in both cases a major peak of T3 in the circulation occurs (37).

Regenerative Approaches

Thyroid transplant was one of the first approaches to treat hypothyroidism, as many patients improved after receiving animal (sheep or goat) or human thyroid glands (from patients with Graves' disease or goiter) (4). Despite early successes this form of treatment was abandoned as symptoms of hypothyroidism kept recurring. More recently, a method was described that generate thyroid cells from stem cells by overexpressing the transcription factors NKX2.1 and PAX8 in mouse embryonic stem-cells (mESC) (58). This leads to fully differentiated follicular cells that express thyroid-specific markers such as TSH receptor, sodium/iodide symporter NIS and thyroglobulin. When exposed to human TSH, these cells organize with a three dimensional follicular architecture. These structures were next grafted under the kidney capsules of hypothyroid mice to examine their *in vivo* functionality. Serum FT4 became detectable after 4 weeks of transplant along with a reduction in serum TSH levels when compared to levels before grafting (58). Similar experiments were performed by other groups and, as a whole, it is clear that hypothyroid mice can be made euthyroid by transplanting functional thyroid follicular constellations (59). Whether this will ever be feasible to be utilized in humans remains to be seen. Patients complaining of persistent hypothyroidism symptoms could benefit from a new thyroid gland and physiological secretion of both T3 and T4.

Tissue Targeting of LT3

Substantial progress on LT3 delivery technology has been achieved recently with the development of tissue-specific targeting of T3 molecules. For example, engineered chemical conjugates of glucagon and T3 enabled delivery of T3 to the liver (60). Treatment with this conjugate corrected hyperlipidemia, steatohepatitis, atherosclerosis, glucose intolerance, and obesity in mouse models of obesity. Liver-directed T3 action spared the cardiovascular system from adverse T3 effects. These findings support the therapeutic utility of integrating T3 and a second

hormone into a single molecular entity in order to obtain tissue-specific effects of T3 (60).

Nanotechnology, which involves manipulation of matter sized from 1 to 100 nm, is another tool that has been utilized to achieve tissue-specific delivery of LT3. Nanoparticles containing LT3 have been manufactured using poly(ethylene imine) (PEI) complexed with dodecanoic acid (PEI-C12) with a lamellar nanostructure and a repeat unit of 2.9 nm. In this context PEI-C12 functions as a guest matrix that dissolves LT3 without crystallization (61). This approach was used to test the hypothesis that efficacy of T3 delivery to the brain can be enhanced by encapsulation in nanoparticulate vehicles (62). T3 was encapsulated in poly-(lactide-co-glycolide)-polyethyleneglycol (PLGA-b-PEG) nanoparticles that were coated with glutathione, an efficient means of brain targeted drug delivery. Efficiency of T3 delivery was tested by measuring its ability to protect against ischemic damage in middle cerebral artery occlusion model of ischemic brain stroke. Indeed, administering T3 in nanoparticulate form resulted in significant benefit over injection of a LT3 solution (62).

CONCLUSION

LT3 is commercially available as sodium salt packaged in tablets that result in rapid duodenal availability and absorption (**Table 1**). The development of slow release formulations that could be used in humans has been in the works for years. Claims of slow release LT3 formulations based on changing the composition of the tablets have not been confirmed in clinical trials or have only minimally affected Cmax and Tmax. A number of new strategies and compounds are being explored but details aren't always available due to commercial interests and protection of intellectual property. So far, T3-S and PZL have shown promising results in animal models but formal phase-1 clinical trials have not been conducted. There is no doubt that the momentum around developing new delivery methods of LT3 for humans is building. This will pave the road to better understand and evaluate the use of LT3/LT4 combination therapy and possibly improve patients' quality of life.

AUTHOR CONTRIBUTIONS

TI performed a bibliographic search and compiled a draft of the manuscript. JP and TP worked on the section on PZL and metal coordination. AB coordinated all work and edited the whole manuscript.

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REVIEW
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Novel Insight Into the Epigenetic and Post-transcriptional Control of Cardiac Gene Expression by Thyroid Hormone

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Thyroid hormone (TH) signaling is critically involved in the regulation of cardiovascular physiology. Even mild reductions of myocardial TH levels, as occur in hypothyroidism or low T3 state conditions, are thought to play a role in the progression of cardiac disorders. Due to recent advances in molecular mechanisms underlying TH action, it is now accepted that TH-dependent modulation of gene expression is achieved at multiple transcriptional and post-transcriptional levels and involves the cooperation of many processes. Among them, the epigenetic remodeling of chromatin structure and the interplay with non-coding RNA have emerged as novel TH-dependent pathways that add further degrees of complexity and broaden the network of genes controlled by TH signaling. Increasing experimental and clinical findings indicate that aberrant function of these regulatory mechanisms promotes the evolution of cardiac disorders such as post-ischemic injury, pathological hypertrophy, and heart failure, which may be reversed by the correction of the underlying TH dyshomeostasis. To encourage the clinical implementation of a TH replacement strategy in cardiac disease, here we discuss the crucial effect of epigenetic modifications and control of non-coding RNA in TH-dependent regulation of biological processes relevant for cardiac disease evolution.

Keywords: hypothyroidism, low T3 state, cardiac disease, epigenetic regulators, microRNAs, long non-coding RNAs, T3 replacement, combination therapy

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INTRODUCTION

Remodeling of cardiac tissue architecture is the common denominator of a variety of cardiovascular pathologies including myocardial infarction, coronary artery disease, hypertension, cardiomyopathy, and arrhythmias (1). These heart stressors trigger similar adaptive responses leading to hypertrophy of the remaining cardiomyocytes and activation of cardiac fibroblasts and endothelial cells. Although the physiological purpose of these coordinated cellular events is to repair damaged tissue and preserve cardiac performance, persistent activation of the wound healing process is detrimental and facilitates the progression to heart failure (HF). At the molecular level, HF evolution is characterized by a maladaptive down-regulation of the adult cardiac muscle proteins isoforms such as alpha myosin heavy chain (αMHC) and sarcoplasmic reticulum Ca^{2+}-ATPase 2a (Serca2a) and a concomitant up-regulation of the fetal genes such as beta myosin heavy chain (βMHC) and phospholamban (Pln) (2).

The adult-to-fetal gene program switch is paralleled by a reduction of the circulating and cardiac levels of 3,5,3′-triiodothyronine (T3), the main biologically active TH. Such a low T3 state condition (LT3S) is considered an independent factor of poor prognosis both in acute and chronic heart disease and is believed to exert a key role in pathological cardiac remodeling and HF evolution (3–5). In line with these observations, maintenance of TH homeostasis in the setting of cardiovascular diseases (CVD), has been proven to be anti-remodeling and promote cardioprotective effects by affecting several aspects of cardiac physiology including contractility, energy production, myocyte geometry, and fate, as well as angiogenesis and matrix remodeling (6–9).

It is now clear that THs exert their effects at multiple levels. Increasing evidence points at TH-sensitive epigenetic and post-transcriptional mechanisms as main triggers of the cellular phenotypes that drives cardiac remodeling (see **Figure 1**). Epigenetic refers to all changes at the nuclear DNA level, that control DNA structure or conformation without affecting its sequence. The most common epigenetic modifications include post-translational histone modifications, DNA methylation, and non-coding RNA transcripts (10–12). Among these mechanisms, hystone modifications and DNA methylations are able to dynamically alter gene expression by modulating chromatin accessibility to transcription factors and coregulators. In particular, opposite alterations of chromatin structure play a crucial role in the switch of cardiac protein isoforms observed during development, in pathological cardiac hypertrophy and in response to altered TH levels (13–18).

TH-sensitive non-coding RNAs are also involved in silencing and activation of gene expression programs relevant to heart physiology and pathophysiology. It is now appreciated that only around 2% of human transcripts are translated into proteins. Many of the remaining non-protein coding transcripts play a pivotal functional role in cardiac tissue homeostasis, which makes them critical targets of therapeutic strategies. The best characterized non-coding RNA species, microRNAs (miRNAs), are 22–23 nucleotide long molecules that turn off gene expression by blocking the translation or inducing the degradation of target transcripts. miRNAs have been involved in a broad range of pathophysiological processes underlying cardiac contractility, cell fate, response to oxidative stress and myocardial fibrosis (19–24). Long non-coding RNAs (lncRNAs), another emerging class of non-coding RNAs, have a length >200 nucleotides. The abundance of lncRNAs in the cardiovascular system is consistent with their crucial role as part of complex regulatory networks governing heart physiology and pathology (25–27). Of particular interest is the recent concept that a cross-talk between lncRNAs and remodelers of chromatin structure may participate in the regulation of cardiac geometry (28, 29).

Increasing experimental evidence suggests that the above described TH-sensitive epigenetic and post-transcriptional mechanisms are critical triggers of maladaptive cardiac remodeling and HF evolution. To reinforce the importance of maintaining TH homeostasis in the context of cardiac injuries, this review is aimed to comprehensively dissect the contribution of epigenetic modifications and non-coding RNAs to the cardioprotective action of T3.

Processes	Genes	Epigenetic action
Myosin switch	αMHC	H3acetyl; H3K4met mir-208a LncMyheart Brg1-dependent gene repression
	βMHC	H3K9met mir-208b Brg1-dependent gene expression
Calcium handling	Serca2a	H3acetyl
	Pln	H3acetyl; H3K4met
Oxidative metabolism	Pgc1α	Sirt1-dependent activation
	Cpt2	mir-222; mir-31; mir-155
Antioxidant defences	SOD2	Sirt1-dependent activation mir-222; mir-31; mir-155
Cell fate	P53 cascade	Sirt1-dependent activation mir-30; mir-144/499
Antifibrotic action	TGFβ1	mir-29; mir-133; mir-30
	CTGF	mir-29; mir-133; mir-30
	MMP2	mir-29; mir-133; mir-30
Cardiac T3 synthesis	TPO	DNA-met mark remodeling

FIGURE 1 | Sketch of the main T3-modulated processes with the corresponding epigenetically regulated genes. In red: genes and epigenetic mechanisms activated by T3. In blue: genes and epigenetic mechanisms inhibited by T3.

CHROMATIN REMODELING IN CARDIOVASCULAR DISEASE: AN OVERVIEW

The function and organization of the genome is dynamically regulated by numerous epigenetic mechanisms that ensure a rapid integration of different signals or inputs (30). Among these mechanisms, post-translational histone modification is crucial to all genome-based activity. Histone proteins are the basic packer and arranger of chromatin structure and can be modified by various post-translational modifications to alter DNA accessibility and gene expression (31). The dynamic transitions between different chromatin conformations rely on a balance between changes that favor a silent state and those that foster a transcriptionally active state (30). In general, histone deacetylation via histone deacetylases (HDACs) allows

DNA to wrap around histones more tightly, thus inducing a repressive state. On the contrary, histone acetylation via histone acetyl transferases (HATs) induces chromatin relaxation and gene transcription. Abnormal enrichment of these epigenetic marks move the balance between open and closed chromatin conformations, resulting in an altered transcriptional activity that may favor cardiac disease evolution (32).

For example, an increase of HDAC enzymes conveys the cardiac stress signals to the pathological pro-growth gene program observed in *in vivo* and *in vitro* models of cardiac hypertrophy and adverse remodeling. Along this line, HDAC inhibitors have proved efficacious in several pre-clinical models of HF (33), leading to a reduction in ischemic/reperfusion (IR) injury and post-ischemic remodeling (34–37), hypertrophy (38–43), fibrosis (44, 45), and inflammation (46, 47). On the other hand, abnormal hystone acetylation via p300 HAT has been involved in salt-induced hypertensive HF (48) and agonist-induced cardiac hypertrophy (49).

Histone methylation, another widespread type of chromatin modification, plays important roles in cardiac lineage specification and differentiation (50, 51), as well as in heart development (52, 53), and pathogenesis of congenital and acquired heart diseases (54, 55). In general, histone 3 methylations at lysine 9 (H3K9) and 27 (H3K27) mark regions of transcriptionally inactive heterochromatin, while histone 3 methylations at lysine 4 (H3K4), 36 (H3K36), and 79 (H3K79) are usually associated with transcriptionally active euchromatin (56). A current model suggests that some methylation marks may dynamically change in response to developmental, environmental or cellular stress cues. Methylated histones are then recognized by chromatin regulators that recruit other factors to alter the promoter accessibility (57). Several histone metyltranferase (HMT) and histone demethylase (HDM) have been identified so far that are differentially expressed during cardiomyocytes differentiation and that are involved in the fetal re-programming of CVD (56).

A further important epigenetic mechanism is the methylation of the DNA cytosine residues at the CpG dinucleotide sequences (58). This epigenetic modification is typically associated with gene silencing, although its effect may be dependent on its location with respect to the target gene. DNA methylation is catalyzed by DNA methyltransferases (DNMTs) (59); of the three DNMTs, DNMT1 is responsible for the maintenance of stable methylation patterns, whereas DNMT3a and DNMT3b catalyze *de novo* methylation (59).

Comprehensive studies indicate that alterations in DNA methylation profiles contribute to the orchestration of biological processes involved in CVD. For example, in HF patients altered methylation marks have been found in promoter of genes driving myocyte apoptosis, fibrosis and contractility (60). Targeted DNA methylation profiling of cardiac tissue from patients with dilated cardiomyopathy revealed hypomethylation and significantly elevated gene expression of matrix metalloprotease 2 (MMP2) and connective tissue growth factor (CTGF), two genes important for the turnover and stability of the extracellular matrix (ECM) (61). In human ischemic cardiac disease, a genome-wide DNA methylation analysis integrated with RNA sequencing evidenced a transcriptional reprogramming that repressed oxidative metabolism by promoter hypermethylation of genes involved in mitochondrial respiration, kreb cycle, and fatty acid beta oxidation (62). On the contrary, anaerobic glycolytic genes were found to be hypomethylated, altogether depicting a regression to the fetal gene program of substrate utilization (62).

Overall, the above-described findings indicate that a better understanding of the epigenetic mechanisms underlying the regulation of chromatin scaffold may open a new therapeutic avenue to blunt pathological cardiac growth and progression to HF. At this regard, T3 has been found to play a key role in maintaining the adult cardiac gene expression program via recruitment of chromatin remodeling determinants at TH receptors (THR). Therefore, restoring the physiological TH homeostasis under hypothyroidism or LT3S condition may contrast the pathological cardiac gene re-programming.

T3-DEPENDENT REGULATION OF GENE EXPRESSION REQUIRES HYSTONE MODIFICATIONS

THR transcription factors belong to the steroid hormone receptor super-family and are encoded in two genomic loci that produce two different isoforms (THRA and THRB) (63, 64). The THRA1 and B1 isoforms are widely distributed and exhibit overlapping patterns of expression (64). However, the THRA1 is mainly expressed in skeletal and cardiac muscle while the THRB1 isoform is more abundant in liver, kidney, brain, and thyroid (63, 64). The use of knock-in animal models harboring THR mutations has provided helpful insight to deciphering the specific role of THRA and B isoforms and distinguishing between canonical and non-canonical cardio-metabolic action of T3. A complete description of this literature is beyond the scope of this review the interested readers are referred to recent papers (65, 66). Briefly, THRA plays a crucial role in post-natal development and cardiac function, whereas THRB is mainly involved in controlling liver metabolism, the hypophysis pituitary thyroid axis, the circulating levels of TH, and the development of the retina and the inner ear (65). In addition, mice expressing mutant THRs that cannot bind DNA provided *in vivo* evidence that relevant physiological effects mediated by THRs are independent from DNA binding and direct activation of gene expression. These so-called non-canonical TH signaling includes energy metabolism, locomotor activity, and heart rate (66).

When acting through the canonical pathways, THR form homodimers or heterodimers with retinoid X receptor and bind to specific THR response element (TRE) in the regulatory region of the target genes. In contrast to other receptors of the family, unliganded THR localize to nucleus and interact with nucleosome-embedded DNA (67, 68). It has long been recognized that THR are master regulators of the chromosomal structure in driving transcriptional activation or repression (69). THR regulation of gene expression is commonly described by a model of chromatin folding switch (63). On T3-positively regulated target genes, unliganded, chromatin-bound THRs

interact with transcriptional co-repressors such as Silencing Mediator of Retinoic acid receptor and TH receptor (SMRT) and Nuclear Corepressor- 1 (NCoR1) and represses transcription through HDACs recruitment (70–74). Binding of the hormone is supposed to induce a conformational change of THR, leading to detachment of corepressors and binding of coactivators such as Steroid Receptor Coactivator 1 (Src1) or TH Receptor-Associated Protein (TRAP) (75–78). In turn, coactivators recruit p300 HAT and form an open status of chromatin that allows the binding of polymerase II transcriptional machinery and the activation of target gene expression (63, 79–89).

This mechanism of action has important physiological implications in presence of TH dyshomeostasis. At low TH concentrations, as in the presence of hypothyroidism, or low T3 state, the unliganded THR is expected to suppress transcription instead of operating as an inactive, passive receptor. Consistently, hypothyroidism models present a much more sever phenotype than THRA and THRB double knockout mouse model (81–84).

The mechanism of transcriptional regulation at negatively regulated THR targets, that are repressed by T3 binding and activated by unbound THR, is less understood (85). It has been proposed that the corepressors and coactivators act in an opposite manner on this kind of target genes, but the exact underlying molecular mechanism is still unclear (85, 86). Genome-wide profiling of THR binding sites in experimental model of hypo- and hyperthyroidism highlighted additional mechanisms of positive vs. negative regulation of gene expression by T3 (63, 87). These studies demonstrated that: (1) a considerable number of intronic and intergenic regions exists where THR are dynamically recruited upon T3 binding to activate transcription; (2) different DNA binding motifs are preferentially enriched at T3 activated or repressed target sites; (3) T3-facilitated gene repression is associated to reduced chromatin accessibility or decreased THR occupancy (63, 87). In the latter case, transcriptional down-regulation could be mediated by reduced affinity of the T3-liganded THR for specific DNA binding motifs (87).

Notwithstanding being recently revisited and implemented (63, 87), the bimodal switch model of THR-mediated chromatin remodeling still holds true for the antithetical regulation of the myocardial MHC, Serca2a, and phospholamban gene expression observed during cardiac development and disease (15–18, 88–90) (see **Figure 1**). A role of differential histone acetylation in T3 regulation of myocardial αMHC gene expression was first demonstrated in a hypothyroid rat model (15). In that study, treatment with the HDAC inhibitor trichostatin A (TSA) was unable to switch the MHC expression in the absence of T3. However, TSA potentiated the activation of the αMHC gene when administered along with T3, supporting the hypothesis that an altered histone acetylation is involved in the T3-mediated regulation of MHC gene transcription. Chromatin immunoprecipitation subsequent studies confirmed that TH levels induced specific histone modifications markers at the α and βMHC promoter locus with opposite effect on MHC isoform expression (16–18) (see **Figure 1**). In particular hypothyroidism determined a reduction of the H3 acetylation and H3K4me marks at αMHC promoter, along with an increase of H3K9met at the same locus. Also, hypothyroidism enhanced βMHC expression by increasing H3 acetylation and H3K4me at the βMHC locus. Reversal of the hypothyroid condition restored the marks of active chromatin on αMHC while repressing them at the βMHC promoter (16–18) (see **Figure 1**). Interestingly fetal or pathologically-stressed heart showed similar epigenetic marks at the repressed αMHC promoter as observed under hypothyroidism (14). Such outcome requires the activity of the ATP-dependent chromatin remodeler Brahma-Related Gene 1 (Brg1). Brg1 is known to govern two independent pathways that drive cardiac growth and differentiation during development and in pathological condition. In the fetal life a high level of Brg1 maintains myocytes in an embryonic state, with inhibition of αMHC expression and activation of βMHC (12). During cardiomyocyte differentiation Brg1 expression is significantly reduced, facilitating the switch to αMHC expression (12). A re-activation of Brg1 is observed in the adult heart of cardiac patients or experimentally stressed animal models, and drives the hypothyroid-like epigenetic events leading to the pathological α to βMHC switch (14). The opposite effects of T3 and Brg1 in the epigenetic control of MHC isoform expression, along with the different myocardial level of T3 in the fetal and adult heart, suggest an intriguing novel mechanism whereby T3 may affect αMHC myocardial content via antagonizing the Brg1 activity (see **Figure 1**).

Epigenetic modification is also required for the T3-dependent activation of Serca2a, another important player of myocardial contractility (88) (see **Figure 1**). Serca2a is a critical determinant of the Ca^{2+} uptake within the sarcoplasmic reticulum (SR) thus affecting the excitation–contraction coupling. Reduced expression of Serca2a has been widely reported in both systolic and diastolic HF (91–93) while Serca2a overexpression was documented to ameliorate cardiac performance and to reduce the incidence of ventricular tachyarrhythmias (94–96). Therefore, enhanced Serca2a expression is considered a potential master plan to blunt cardiac dysfunction and arrhythmias. However, efficacious pharmacological interventions to restore Serca2a levels are not available, and adenoviral-mediated gene delivery of Serca2a in HF patients failed to rescue the reduced ejection fraction (97). As for αMHC, Serca2a expression at mRNA and protein levels increases after birth in parallel to a surge of T3 level. The decreased expression of Serca2a observed in hypothyroidism can be normalized by restoring the hormonal levels through TH administration (98). In an *ex vivo* study on human myocardial biopsies, T3 replacement at physiological doses rescued the intracellular flux of Ca^{2+} that was compromised following long-term T3 deprivation (99). Thus, it is plausible that the LT3S, observed during cardiac disease evolution, may promote Serca2a repression. In line with this interpretation, at Serca2a promoter the liganded THR recruit p300 HAT and TRAP regulators in sequential steps to induce hystone acetylation and increase protein synthesis (88).

Serca2a activity is reversibly inhibited by the phosphoprotein Pln, thus modulators of the Serca2a/Pln regulatome are important determinant of Ca^{2+} cycling kinetics and cardiac contractility (100). It is clearly established that T3 is able to suppress the Pln gene expression in the heart (101–104). There is a tight correspondence between the entity of

Ca^{2+} uptake within the SR and the Pln to Serca2a ratio in hypothyroidism, euthyroidism, and hyperthyroidism conditions, which determines the cardiac performance (103). The precise mechanism of T3 action on Pln remained unclear until the study of Belakavadi and coworkers (90). These authors revealed that T3-dependent down-regulation of Pln in cardiac myocytes is mediated by THRA1 and involves the hormone-directed recruitment of HDAC and HDM activities to decrease both histone H3 acetylation and H3K4met epigenetic marks (90) (see **Figure 1**).

T3 AND DNA METHYLATION

In contrast to histone modifications, the effect of TH on DNA methylation is less well-studied. The available data are limited to extra cardiac tissues and suggest the involvement of TH in the regulation of DNA methylating enzymes. Persistent exposure of neonatal rats to antithyroid drug propylthiouracil leads to considerable changes of DNMT expression with increased induction of oxidative stress and up-regulation of p53 in adult liver (105, 106). Newly diagnosed hyperthyroid patients had genome-wide hypomethylation and lower DNMT1 expression in T and B lymphocytes; while relief of hyperthyroidism with antithyroid drugs restored the global DNA methylation and DNMT1 expression (107). These findings raise the intriguing working hypothesis that differential DNA methylation in circulating blood cells could be exploited to stratify patients according to their thyroid state. If validated by dedicated studies, such an approach could be particularly useful to guide better personalized treatment even in patients with milder alteration of thyroid homeostasis such as LT3S.

The role of TH in the direct modulation of DNMT expression has been extensively analyzed in processes that require global changes of tissue architecture such as Xenopus metamorphosis and post-natal neurological development. A recent study showed that the critical gene for *de novo* DNA methylation, DNMT3a, is controlled by T3 in developing frog tissues. The expression of DNMT3a increased as endogenous plasma T3 rises, and could be induced by exogenous T3 administration (108). The same authors also found that the post-natal surge of T3 levels in mouse brain was paralleled by increased expression of DNMT3a and that the treatment of mouse neuronal cells with T3 caused rapid induction of DNMT3a mRNA (109). Based on these findings it has been proposed that T3 modulation of DNMT3a may represent an evolutionarily conserved mechanism for regulating the post-natal brain differentiation by inducing genome-wide changes in DNA methylation marks. As well as the brain, the heart is a terminally differentiated organ whose post-natal maturation requires a surge of T3 circulating level. Therefore, it can be speculated that similar T3-mediated epigenetic DNA modification as in the brain may be at work to determine heart differentiation. Along this line, it is also conceivable that the LT3S state observed in CVD evolution may favor altered DNA methylation underlying the regression to the fetal gene program. Unfortunately, whether T3-dependent DNA methylation plays a role in cardiac pathophysiology remains an open question that deserves further investigations.

There is, however, emerging evidence indicating that altered DNA methylation may be involved in the setting of myocardial LT3S in patients with CVD. By using RNA-sequencing, it was recently demonstrated that the human myocardium expresses the machinery for TH biosynthesis (110, 111). In patients with ischemic cardiomyopathy, the mRNA levels of thyroperoxidase (TPO), a key component of this TH enzymatic machinery, was reduced in association with reduced T3 levels and altered methylation pattern of the TPO gene (see **Figure 1**). Also, retrieval of data from methylation analyses in vessel from atheroschlerotic patients confirmed similar methylation pattern in the same CpG sites, thus suggesting that altered methylation marks at TPO gene may contribute to the reduced local production of T3 observed in CVD (110).

Finally, an indirect route by which T3 might regulate cardiac DNA methylation, is via interaction with the sympathetic nervous system (SNS). It is well-established that T3 can alter the responsiveness to sympathetic stimulation by enhancing the function and density of the adrenergic receptors within the cardiovascular system. The available data also indicate that activation of adrenergic signaling modulates epigenetic mechanisms of DNA methylation implicated in cardiac hypertrophy and HF (112, 113). These intriguing premises lay the ground for future studies aimed at elucidating the cross-talk between T3 and the SNS in the modulation of cardiac DNA methylation in physiological and pathological conditions.

T3 REGULATION OF CARDIAC MICRORNAS

Regulation of myocardial miRNA is an emerging mechanism for T_3-mediated epigenetic control of cardiac gene expression. As single miRNAs can target several genes within functional related biological processes, controlling the expression of one microRNA can affect whole gene networks and alter the phenotype of complex diseases (20). Accordingly, T3-regulated miRNAs have been shown to counteract many noxious processes underlying adverse cardiac remodeling.

A recent miRNA profiling in hearts of hypothyroid and hyperthyroid mice revealed a signature of T3-responsive miRNAs, including miR-208a, that are predicted to repress the transduction of the prohypertrophic, pathological cascade (114). miRNA-208a is one of the main heart-enriched miRNA that plays a key role in cardiac physiopathology, it belongs to a miRNA family that also comprises miR-208b and its promoter is located in an intronic region of the αMHC gene. Within the heart, miR-208a and miR-208b are implicated in the MHC isoform switch during heart development and disease. In mice hearts physiological levels of miR-208a are necessary for a correct cardiac electrical activity (115). Conversely, cardiac overexpression of miR-208a causes pathological cardiac hypertrophy by targeting TRAP1 and myostatin, that are negative regulators of muscle growth (115). Reduction of myocardial TH levels in cardiac disorders favors αMHC and miR-208a repression while administration of THs to cardiomyocytes in culture significantly upregulates the αMHC/miR-208a expression-ratio and decreases the βMHC/miR-208b expression-ratio (115) (see

Figure 1). These data add a further level of complexity to the T3-dependent regulation of MHC expression and suggests that physiological TH concentrations are required to ensure proper miRNA levels and to preserve MHC composition.

In experimental models of post-ischemic LT3S, T3 replacement was paralleled by rescued levels of miR-30a expression within the area at risk (116). The miR-30 family members are expressed at high levels in the adult heart, and are significantly down-regulated in human HF, in experimental IR, and *in vitro* after oxidative stress (116–118). miR-30 has been shown to target p53, a well-known activator of mitochondrial apoptosis and necrotic pathways (118–120). Consistently, T3 replacement following cardiac IR was associated to a down-regulation of the p53 signaling along with better preserved mitochondrial function and decreased cell death (116). Also, T3 physiological replacement in hypoxia-stressed neonatal rat cardiomyocytes in culture prevented both miR-30a down-regulation and p53 activation. The protective action of T3 was greatly inhibited by miR-30a knockdown. Collectively, these findings support a novel T3-dependent cardioprotective mechanism that influences mitochondrial function and is driven by the miR-30a/p53 axis (116) (see **Figure 1**).

T3 is also involved in the regulation antifibrotic process following cardiac IR stress via up-regulation of miR-29, -30c, and -133 (121). A decrease of these miRNAs following myocardial damage leads to fibrosis and conduction alterations via up-regulation of an array of proteins involved in ECM remodeling (21, 24, 122–124). It has been shown that a LT3S in the early phase of the post-IR setting represents a permissive condition for a long term maintenance of high levels of transforming growth factor beta 1 (TGFβ1) (121). In turn, raised TGFβ1 levels inhibited the antifibrotic miR-29c, miR-30c, and miR-133a and de-repressed their profibrotic targets MMP2 and CTGF. Ultimately, these early molecular events led to adverse remodeling and reduced cardiac contractility (117). LT3S correction through timely T3 infusion hampered the inhibitory effect of a protracted TGFβ1 up-regulation on the antifibrotic miRNA pathway, thus decreasing fibrotic tissue deposition, scar size, and heart dysfunction (121) (see **Figure 1**).

A recent comprehensive miRNA profiling provided novel indication that an early post-IR T3 treatment associates to the modulation of a host of myocardial miRNAs critically implicated in the orchestration of heart development, function, and disease (122). Among the newly identified miRNAs that are up-regulated by T3, the miR-144/451 cluster and miR-499 have documented protective effects in the infarcted myocardium by limiting cardiomyocyte death and excessive mitochondrial fission (125, 126). On the other hand, miR-31, -155, and -222, that are down-regulated by T3, have been shown to promote cardiac dysfunction and adverse remodeling in both HF and ischemic heart disease (127–130). In accordance, the computational integration of the T3 differentially expressed mRNAs and T3 differentially expressed miRNAs identified a network of T3-modulated regulatory circuitries (122). Such connections are predicted not only to inhibit the harmful signaling cascade leading to cardiomyocyte loss, mitochondrial alterations, inflammation and extracellular matrix remodeling, but also to potentiate protective pathways that preserve mitochondrial quality control and oxidative metabolism including mitochondrial fusion, protein import and folding, mitochondrial antioxidant activity, and carnitine shuttle (122) (see **Figure 1**). Such data are in agreement with the notion that T3 plays a pivotal role in cellular growth, homeostasis and metabolism (131, 132).

The available evidence on experimental models indicates that the protective cardiac action of T3 is under the synergistic control of T3-responsive miRNAs. Also, a protracted post-ischemic LT3S seems to be a permissive condition for the maintenance of the fetal miRNA program. Such a fetal gene recapitulation contributes to the pathological changes associated with progressive cardiac dysfunction (117, 133). Indeed, in pathological hypertrophy, a striking analogy has been found between the miRNA expression profile found in human HF and that observed in the hearts of 12–14 weeks old fetuses (23). Therefore, based on the published data, a model can be envisioned in which stress stimuli alter the intra-cardiac TH signaling leading to reduced T3 level. In this context, maintenance of T3 cardiac homeostasis through T3 replacement might blunt the dysregulation of T3-dependent miRNAs and their target mRNAs thus limiting adverse remodeling.

T3 REGULATION OF CARDIAC LONG NON-CODING RNA

LncRNAs, another class of non-protein coding DNA products, have been shown to play a major role in many aspects of myocardial gene regulation, especially the epigenetic processes that underpin organogenesis and remodeling of cardiac architecture (134). In particular, lncRNAs have been proposed to bind certain methyltransferases and demethylases and to guide them to specific genomic regulatory region or to inhibit their interaction with the chromating scaffold and DNA (134). Among the various species of lncRNA, the natural antisense transcripts (NATs) form an abundant class of regulatory molecules that are transcribed by the opposite strand of protein coding genes. One of them is located within the 4.5-kb intergenic space between the αMHC and βMHC genes and is implicated in the regulation of the MHC switch (135). Within this intergenic region a bifunctional promoter has been found to coordinately control the transcription of the αMHC sense mRNA and βMHC antisense RNA (136). The βMHC NAT extend over the βMHC gene and its promoter. Stimulation of such intergenic transcription has been supposed to silence βMHC sense transcription via RNA-RNA interference to favor the αMHC isoform expression (135, 136). Compelling evidence in hypothyroid and hyperthyroid models indicate that T3 is a positive regulator of this NAT within the myocardial tissue (16, 137). It has been suggested that the T3-dependent NAT binds chromatin repressing complexes such as HDAC and HMT to alter chromatin accessibility at the βMHC promoter (16). According to this model, in pathological condition, reduced T3 levels repress the intergenic NAT along with αMHC gene

transcription while relieving the repression at βMHC gene, which allows a rapid α-to βMHC switch (16).

Recently a lncRNA, named Myheart (Mhrt), located in the antisense region between the αMHC and βMHC has been found to protect the heart against pathological hypertrophy (28). This action seems not to involve RNA–RNA sequence interference between Mhrt and the βMHC transcript as previously supposed. Instead, Mhrt binds to and represses the activity of Brg1 protein, the chromatin remodeler that triggers the fetal gene reprogramming of cardiac myopathy (28). Following pathologic cardiac stress, the Brg1-HDAC chromatin repressor complex is up-regulated leading to the inhibition of Mhrt transcription and development of cardiomyopathy, which is prevented by reestablishing the pre stress levels of Mhrt (28).

INDIRECT TRANSCRIPTIONAL REGULATION BY THS VIA SIRTUIN MEDIATED EPIGENETIC MECHANISMS

Sirtuins (Sirt) are a family of NAD^+-dependent deacetylating enzymes. Sirt1, the best characterized one, coordinates the metabolic adaptation of the whole organism by modulating the transcription pattern in response to the cell need (138). Several epigenetic mechanisms have been so far identified for Sirt1-mediated regulation of gene expression. Sirt1 can lead to transcriptional repression through direct histone deacetylation, or via methylation of histones and DNA (138). Secondly, Sirt1 can bind and deacetylate a wide range of transcription regulators including TR, thus affecting the expression of target genes positively or negatively (138). Also, Sirt1 deacetylates histone-modifying enzymes to regulate their function. In this manner, Sirt1 interacts with p300 HAT and inhibits its enzymatic activity to promote nucleosomal histones hypoacetylation and alter gene expression outcomes (139).

Sirt1 plays a critical role in CVD physiopathology being involved in the regulation of several cellular metabolic pathways as well as in the death or survival decision making (140). For this reason, Sirt1 is the most extensively studied sirtuin in the cardiovascular system (140). The available literature indicates that Sirt1 can exert beneficial or noxious cardiac effects in a dose dependent manner (140–142). In mouse models of cardiac IR a mild to moderate Sirt1 overexpression (up to about sevenfold) proved cardioprotective by decreasing the pro-apoptotic cascade and increasing the mitochondrial antioxidant defenses (141). On the contrary, excessive Sirt1 up-regulation (more than 10 fold) elicits opposite effects including mitochondrial dysfunctions and low energy production associated to decreased expression of the peroxisome proliferator activated receptor gamma coactivator 1 alpha (PGC-1α) (142). These data indicate that to afford cardioprotection the levels of Sirt1 must be rigorously controlled (140).

TH state has been shown to affect Sirt1 activity either directly, by affecting the protein levels, and indirectly, by influencing the cytosolic concentration of NAD^+ that is generated through oxidative phosphorylation (143, 144). Also, Sirt1 is required to promote THR-mediated gene expression. In liver tissue, Sirt1 and THRB1 interact with each other to enhance T3-responsiveness (145, 146). Accordingly, T3 has been reported to share many metabolic mechanisms with Sirt1 (147, 148) (see **Figure 1**). Even if the T3-Sirt1 connection has been extensively characterized only in extra cardiac tissue, we recently found that T3 regulates the same antioxidant, antifibrotic and prosurvival cardioprotective mechanisms as Sirt1 (122, 142, 149–151), thus suggesting that the T3-Sirt1 cross-talk may be a generalized mechanism to increase the panel of T3-regulated processes under different patophysiolgical conditions. Finally, it is worth mentioning the emerging cardio-metabolic action of 3,5 diiodothyronine (T2) via Sirt1 deacetylation pathways (152, 153). T2 is an endogenous active metabolite of T3 that is able to modulate similar processes as T3 in many tissues without manifesting all the deleterious cardiac effect of hyperthyroidism, even when used at pharmacological doses (152, 153). One proposed mechanism of action is the rapid activation of Sirt1 that deacetylates Pgc-1α and Sterol regulatory element-binding proteins-1c (Srepb-1c). These transcription factors then trigger a cascade of events improving mitochondrial biogenesis and energy metabolism, while preventing fat accumulation and diet induced insulin resistance (153). These promising findings encourage further studies to directly explore the therapeutic use of T2 under cardiac disease conditions.

FUTURE PERSPECTIVES AND CONCLUDING REMARKS

Overall the available data on T3 cardiac effects should provide helpful guidance to manage hypothyroidism or LT3S conditions optimizing the benefit to risk profile.

Although T3 mediates the vast majority of physiological processes (see **Figure 1**), LT4 is still considered the unique choice to treat hypothyroidism. Unfortunately, due to altered peripheral conversion of T3 from T4, ∼10–20% of patients taking T4 monotherapy experience symptom persistence or recurrence despite normal TH test (154–158). This outcome highlights the need to consider better personalized replacement approaches for a certain patient population. Individualized T3 or combination therapies should be developed to mimic a more physiological intratissutal thyroid hormone signaling. At this regard, the dynamic nature of some epigenetic modifications that are particularly sensitive to altered TH homeostasis, should be exploited by the biomarker discovery research to distinguish patients that may best benefit from T4 monotherapy or combined replacement, thus avoiding the potential adverse effect of thyrotoxicosis.

As a second key point, it has been a long standing dogma that the LT3S in the hyperacute post-IR phase may be beneficial by reducing energy consumption (159). However, increasing clinical and experimental data indicate that the persistence of a LT3S favors cardiac disease evolution and worsens patient prognosis (6, 160, 161). Importantly, to be efficacious against adverse remodeling, the timing of LT3S correction should take into account the regenerative window that is lost in the late post-IR phase (162). Accordingly, T3 administration started 1

week after MI ameliorated heart performance without reversing cardiac remodeling (163). By contrast, THs administered early after IR enhanced cardiac function and prevented left ventricle remodeling (116, 121, 122, 164, 165). These finding are in line with the key role of T3 as main modulator of cardiac repair/regeneration, a process that is critically affected by dynamic epigenetic mechanisms. As for other cardiac epigenetic regulators, such as plant compounds or selected exosomes (166, 167), lower T3 doses proved more efficacious than pharmacological treatments in contrasting the noxious pathways of adverse remodeling (168, 169). Such information on optimized dose and timing for T3 delivery after IR should provide clinically translatable protocol based on inexpensive, commercially available T3.

AUTHOR CONTRIBUTIONS

GI and FF conceived the manuscript. FF has written the manuscript with input from all authors. GN and LP contributed analysis of the bibliographic material. All authors have provided critical discussion and have revised the final manuscript.

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Assessment of the Adequacy of Thyroid Hormone Replacement Therapy in Hypothyroidism

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Background: Recent studies identify a significant number of treated hypothyroid patients who express dissatisfaction with their therapy. At present there are sufficient measures of thyroid function to enable the clinician to establish a diagnosis of thyroid disease with a high degree of sensitivity and specificity. The purpose of this study was to quantitate the use of a new and novel assessment of clinically relevant hypothyroid symptoms in the management of patients with thyroid disease and to identify a tool that could help clinicians to assess adequacy of LT_4 treatment.

Methodology: Unselected outpatients of the Thyroid Clinic of the North Shore University Hospital at Manhasset completed a questionnaire asking them to rate their physical symptoms related to thyroid disease as part of their standard care. This questionnaire consisted of 10 signs and symptoms. The questionnaire was collected from 198 control subjects, 241 subjects with primary hypothyroidism (under treatment), 113 euthyroid subjects (benign nodular thyroid disease), 73 previously hyperthyroid subjects (previously treated), and 27 subjects with thyroid cancer. A repeat questionnaire was obtained from 48 subjects with primary hypothyroidism (20%), 19 euthyroid subjects (17%), and 17 subjects previously hyperthyroid (23%).

Data Analysis: The mean score for the sum of the signs and symptoms in the primary hypothyroid group with no medication change was 9.62 ± 1.29 for the initial questionnaire, and 10.04 ± 1.32 for the follow up questionnaire (not significant). For the primary hypothyroid patients requiring a medication change, at the time of the initial questionnaire the mean serum TSH was 12.86 ± 2.75 mcU/ml. Concurrently with the normalization of TSH, a statistically significant improvement in the sum of signs and symptoms mean score for this group was noted (16.32 ± 1.93 initial vs. 10.32 ± 1.46 after treatment to normalize TSH).

Conclusion: The proposed newly devised hypothyroid scale correctly identified subjects with TSH elevation and clinical/subclinical hypothyroidism based on their clinical signs and symptoms. In this particular subset of patients, the hypothyroid symptom scale showed a statistically significant improvement in the sum of the signs and symptoms with the normalization of the subjects' thyroid function.

Keywords: symptom scale, T4, T3, treatment, diagnosis of hypothyroidism

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INTRODUCTION

There are a number of thyroid function tests (TFTs), which enable the clinician to establish a diagnosis of thyroid disease with a high degree of sensitivity and specificity. The application of these tests, particularly the TSH, makes the diagnosis of overt thyroid dysfunction fairly straightforward in most cases. There are however notable exceptions in which these laboratory tests may not be a reliable measure of thyroid hormone action in individual patients (1). These conditions include, but are not limited to, secondary hyper and hypothyroidism, subclinical forms of hypothyroidism and hyperthyroidism, thyroid hormone resistance, alterations in thyroid hormone binding by a variety of serum carrier proteins, interactions with different medications such as lithium, amiodarone, and iodine, as well as nonthyroidal (low T_3) illnesses (2–8). In the assessment of these patients, it would be useful to have an additional quantitative clinical measure that both directly and indirectly reflects the cellular action of thyroid hormone and could be used clinically to aid in diagnosis and treatment (1, 8–10). The recent interest in T_4/T_3 combination therapy in patients treated with L-T_4 monotherapy calls out for a quantitative measure of well-recognized hypothyroid symptoms and the response of these symptoms to individualized treatments. Hypothyroid symptom scales have been previously developed and modified over the years to primarily aid in the diagnosis of hypothyroidism (11, 12) to target those patients who would likely be candidates for further testing, but none to date have been designed to assess the adequacy of treatment in patients currently on thyroid hormone replacement with persistent symptoms and to potentially guide novel therapeutic regimens.

Fifty years ago, before the development of adequate and reliable TFTs, Billewicz et al. (11) described a diagnostic index that assessed the presence or absence of various signs and symptoms of hypothyroidism for the purpose of establishing a diagnosis of hypothyroidism. With the development of newer, more reliable TFTs, in 1997 Zulewski et al. (12) revised the hypothyroid signs and symptoms clinical score for individual assessment of the severity of thyroid failure. It was designed based on signs and symptoms originally chosen by Billewicz and a scoring range was determined in patients with untreated overt hypothyroidism and compared to patients with normal thyroid function. Scores were based on the presence (1) or absence (0) of hypothyroid signs and symptoms. Ranges were determined based on average scores in patients and controls and the frequency of these symptoms in the same populations. In addition, a correction factor (+1) was added to a patient's score when his (her) age was <55 years. The diagnostic range for this clinical score was established as ≤ 2 = euthyroid; 3–5 = intermediate; >5 = overt hypothyroid. In overt hypothyroidism the average score was found to correlate with ankle reflex relaxation time, total cholesterol, and creatine kinase. However, while the score correlated with fT_4 and fT_3, it did not correlate with TSH, the gold standard for thyroid function testing. TSH and fT_4 correlated with scores in the middle range. The study concluded that the classical signs and symptoms of hypothyroidism were only present in patients with severe overt hypothyroidism with low serum T_3 levels, but were minimal or absent in patients with normal T_3, despite low fT_4 or in patients with subclinical hypothyroidism (12). Therefore, it seems that T_3 levels were the most reliable predictor of hypothyroid signs and symptoms. Another important feature of both the Billewicz and Zulewski diagnostic tools is that the scoring is accomplished by physicians who function as examiners, and not by a questionnaire administered to the patient.

It has further been demonstrated that physical examination alone in the diagnosis of hypothyroidism cannot confirm or rule out hypothyroidism without including TFTs (13). To assess the significance of clinical vs. biochemical assessment of hypothyroidism in patients to optimize L-T_4 dosing, the Billewicz scale was used and compared to biochemical measures (14). Of almost 400 subjects found to be biochemically hypothyroid, <1 fourth could be classified as hypothyroid based on the Billewicz score.

Contrary to the Billewicz and Zulewski scales, in 1997, Canaris et al. (15) examined a group of hypothyroid patients and compared them to matched controls by self -administered questionnaire. In this study, only newly diagnosed patients with a TSH above 20 μU/mL and decreased T_4 were eligible to participate. The number and percentage of positive symptoms were determined for each patient. Symptoms were scored as present or absent (positive or negative). Using a scale of 1–5, only severe ratings of 4 or 5 were counted as present, ratings of 1–3 were considered absent. Patients were also asked whether the symptom or sign had changed within the past year. For hypothyroid patients, the number of conventional hypothyroid symptoms reported in that group was directly, albeit weakly, correlated to TSH, with a stronger association when more symptoms were reported. Likelihood ratios for the scoring ranges were determined to help assess whether to test for thyroid disease.

Each of the studies described was designed for the purpose of diagnosis of hypothyroidism to aid in deciding whether TFTs are warranted. We have devised a novel hypothyroid symptom scale to assess the adequacy of thyroid hormone replacement therapy. The symptoms were chosen based on those commonly reported to be associated with hypothyroid symptoms that could be self assessed by the patient (10–16). Using this tool, we have identified persistently symptomatic hypothyroid patients who subsequent to appropriate changes in levothyroxine dosages then showed improvement.

METHODOLOGY

Patient Acquisition

Four hundred fifty-four unselected outpatients attending the Thyroid Clinic of the North Shore University Hospital at Manhasset, and 198 control women undergoing routine outpatient mammography were randomly asked to complete a questionnaire asking them to rate their physical symptoms related to thyroid disease. Follow up questionnaires were randomly obtained at the standard 3–6 month return visit to the Thyroid Clinic.

Prior to your visit with the Doctor today, we request that you provide the following information. Next to each symptom, please check the box corresponding to the degree which you have been experiencing that condition for the past month.

Symptom	Absent 0	Minimal 1	Mild 2	Moderate 3	Severe 4
Dry Skin					
Fatigue					
Weight Gain					
Cold Intolerance					
Constipation					
Muscle Stiffness					
Puffiness					
Early Awakening					
Memory Loss					
Feeling Blue					

FIGURE 1 | The hypothyroid signs and symptoms questionnaire. The final 10-symptom questionnaire is the result of refining an initial 12-symptom questionnaire after it was determined that two symptoms have no sensitivity or specificity for hypothyroidism.

Questionnaire

This questionnaire consisted of 10 signs and symptoms including dry skin, fatigue, weight gain, cold intolerance, constipation, muscle stiffness, puffiness, memory loss, feeling blue, and dizziness. The severity of each sign and symptom was rated on a scale from 0 to 4 points: absent (0), minimal (1), mild (2), moderate (3), and severe (4). The item scores were totaled to obtain the overall signs and symptoms score ranging from 0 to 40 points (**Figure 1**).

Data Analysis

Data was collected from 652 patients from the department's patient population. The project was reviewed by the North Shore -LIJ IRB and found to be exempt. Patient and subject consents were not required. The data elements collected included patient age, patient ratings of physical symptoms and thyroid function measurements. For the controls, only age and physical symptoms were collected. Thyroid function measurements included TSH, T_4, and T_3 hormone levels. Patients were divided into groups based on their thyroid disease diagnosis for data analysis. The questionnaire was collected from 241 subjects with primary hypothyroidism (under treatment), 113 euthyroid subjects (benign nodular thyroid disease), 73 previously hyperthyroid subjects, and 27 subjects with thyroid cancer in addition to 198 control subjects with no known thyroid disease. Patients with hyperthyroidism were treated with either ^{131}I or methimazole and thus became hypothyroid requiring replacement therapy. Thyroid cancer patients who have been thyroidectomized were also treated with thyroid hormone replacement therapy. A repeat questionnaire was obtained from 48 subjects with primary hypothyroidism (20%), 19 euthyroid subjects (17%), and 17 subjects who were previously hyperthyroid (23%) (**Table 1**). Data are expressed as the mean ± SE. Statistical analysis of the data obtained was done using SPSS 12.0.1 and Excel software.

TABLE 1 | Number of questionnaires obtained for each group.

	Initial	Follow-up	%
Control	198	0	–
Primary hypothyroid	241	48	20
Previously hyperthyroid	73	17	23
Thyroid cancer	27	0	0
Euthyroid	113	19	17
Total	652	84	–

RESULTS

Table 2 summarizes our findings of all the study groups in this project for the sum of signs and symptoms, TSH, T_4, T_3, and the study subjects' age. The primary hypothyroid group had the highest ratings of symptoms as compared to other patients or controls despite treatment with levothyroxine sodium. Thyroid function tests (TSH, T_4, and T_3) also corresponded to the study groups' diagnoses. Thyroid function tests were not determined in the control study population. The mean age of all study participants was similar except for the thyroid cancer group. The mean of all symptoms in hypothyroid patients was significantly greater (13.6 ± 0.3) compared to all other groups (**Table 2**). The

TABLE 2 | Sum of signs and symptoms, TSH, T_4, T_3 for each group.

Group Diagnosis		Sum of signs and symptoms	TSH (mcU/mL)	T_4 (mcg/dL)	T_3 (ng/dL)	Age (years)
1–Control	Mean ± SE	10.15 ± 0.56	Not Determined			55.83 ± 0.93
	N	198		198		
2–Primary Hypothyroid	Mean ± SE	13.60 ± 0.53	2.16 ± 0.37	9.38 ± 0.19	112.5 ± 3.30	52.16 ± 0.88
	N	241	215	172	85	241
3–Previously Hyperthyroid	Mean ± SE	9.47 ±0.92	0.44 ± 0.12	9.46 ± 0.65	169.1 ± 15.47	52.34 ± 1.92
	N	73	50	29	28	73
4–Thyroid cancer	Mean ± SE	10.93 ± 1.59	0.22 ± 0.11	11.09 ± 1.23	135.4 ± 19.37	45.48 ± 2.51
	N	27	20	19	5	27
5–Euthyroid	Mean ± SE	9.76 ± 0.70	1.43 ± 0.09	8.45 ± 0.28	136.4 ± 6.58	51.58 ± 1.46
	N	113	107	60	35	113

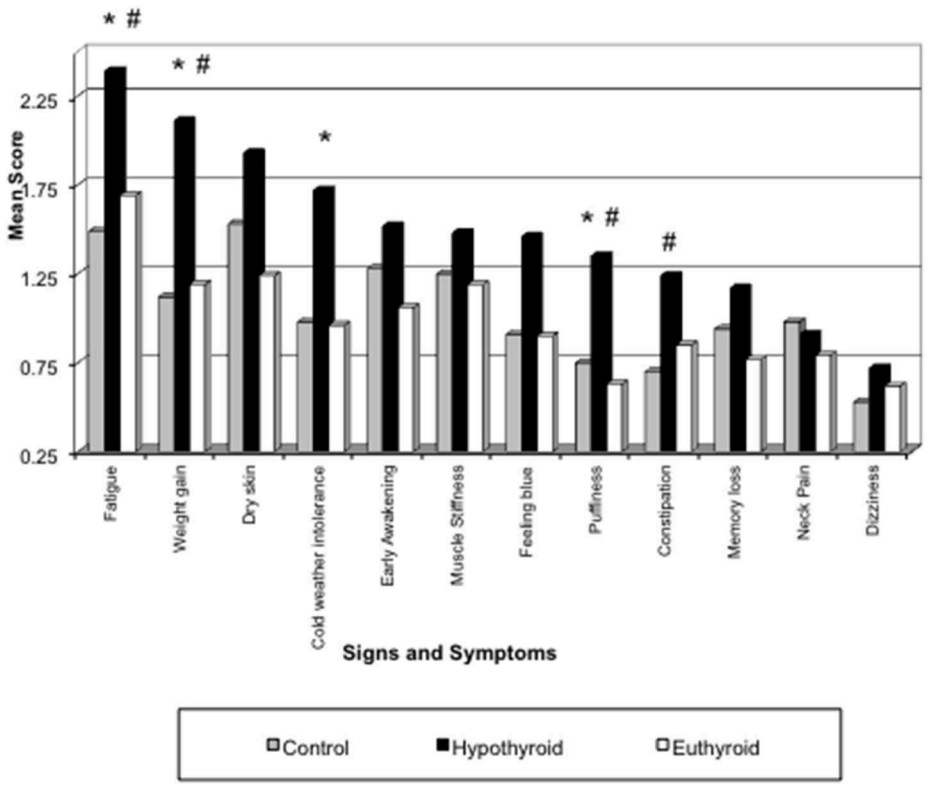

FIGURE 2 | Distribution of signs and symptoms among the study groups. *$p \leq 0.05$ for Hypothyroid vs. Euthyroid; #$p \leq 0.05$ for Hypothyroid vs. Control.

distribution of signs and symptoms among the study groups is shown in **Figure 2**. Significant differences ($p \leq 0.05$) between hypothyroid and euthyroid subjects were found for fatigue, weight gain, cold weather intolerance, and puffiness. Significant differences ($p \leq 0.05$) between hypothyroid and controls subjects were found for fatigue, weight gain, puffiness, and constipation.

Within the primary hypothyroid group of subjects, 48 follow up questionnaires were obtained out of the original 241 study participants. The 48 follow up questionnaires included 22 study subjects who required increased thyroid medication during the study period and 26 study subjects who did not. The analyzed data also included serum TSH, T_4, and T_3 levels, which were obtained at the same time as the initial and follow up questionnaires.

The mean score for the sum of the signs and symptoms in the primary hypothyroid group with no medication change was 9.62 ± 1.29 for the initial questionnaire, and 10.04 ± 1.32 for the follow up questionnaire ($p = 0.517$) (**Table 3**).

For the primary hypothyroid patients requiring a medication change, at the time of the initial questionnaire the mean serum TSH was 12.86 ± 2.75 mcU/ml. With an average increase in levothyroxine from 72.37 ± 10.53 mcg to 100.37 ± 7.67 mcg

TABLE 3 | Test-retest data analysis for the primary hypothyroid group.

A				B			
Primary Hypothyroid without Rx change				**Primary Hypothyroid with Rx increase**			
Test - 1, Retest - 2	**Mean ± SE**	**N**	**Sig.**	**Test - 1, Retest - 2**	**Mean ± SE**	**N**	**Sig.**
Sum of signs and symptoms				**Sum of signs and symptoms**			
1	9.62 ± 1.29	26	NS	1	16.32 ± 1.93	22	0.0002
2	10.04 ± 1.32	26		2	10.32 ± 1.46	22	
TSH (mcU/mL)				**TSH (mcU/mL)**			
1	1.23 ± 0.19	26	NS	1	12.86 ± 2.75	22	0.001
2	1.76 ± 0.48	5		2	1.89 ± 0.35	21	
T_4 (mcg/dL)				**T_4 (mcg/dL)**			
1	9.25 ± 0.39	26	NS	1	7.20 ± 0.61	22	0.01
2	9.20 ± 1.45	4		2	9.76 ± 0.55	20	
T_3 (ng/dL)				**T_3 (ng/dL)**			
1	108.6 ± 5.9	10	NS	1	99.0 ± 11.94	12	0.01
2	136.0	1		2	137.75 ± 7.84	12	
Levothyroxine sodium dose (mcg)				**Levothyroxine sodium dose (mcg)**			
1	95.29 ± 8.01	26	NS	1	72.37 ± 10.53	22	0.034
2	95.29 ± 8.01	26		2	100.37 ± 7.67	22	
Age (years)				**Age (years)**			
1	51.40 ± 2.76	26	NS	1	49.60 ± 2.75	22	NS
2	51.53 ± 2.76	26		2	49.92 ± 2.76	22	

between the initial and follow up points of the study, a statistically significant decrease of the mean TSH to 1.89 ± 0.35 was observed ($p = 0.001$). Concurrently with the normalization of TSH, a statistically significant improvement in the sum of signs and symptoms mean score for this group was noted (**Table 3**) ($p = 0.0002$). As expected, T_4 and T_3 levels increased, but remained within the normal range after the increase of levothyroxine sodium ($p = 0.003$ and 0.017, respectively).

DISCUSSION

The results of our study demonstrated reproducibility and validity of this newly proposed hypothyroid signs and symptoms scale. There was no statistically significant change in the sum of the signs and symptoms in the primary hypothyroid group with no medication change between the initial and follow up visits, suggesting this novel scale's reproducibility. Also, the proposed hypothyroid signs and symptoms scale correctly identified patients with untreated or under treated primary hypothyroidism with the total mean score of 16.74 as compared to the euthyroid and control groups with the total mean scores of 11.31 and 12.40, respectively, suggesting this scale's validity.

Similarly to the results of Canaris et al. (15), our data showed that the symptoms associated with hypothyroidism were predictive of abnormal serum TSH, and the study participants with higher symptoms scores on the hypothyroid scale had significantly higher TSH values. Similarly to the results of Zulewski et al. (12), our hypothyroid scale showed a significantly higher signs and symptoms scores in the untreated or under treated hypothyroid group of patients as compared to the "baseline" score of the control, euthyroid, and adequately thyroxine—replaced hypothyroid study groups.

McAninch et al. (16) conducted a retrospective study that included 99 studies of hypothyroid patients treated with T_4 monotherapy. Previous observations suggested that monotherapy with T_4 may not be adequate because patients complained of persistent symptoms. Results demonstrated that in patients with T_4 monotherapy and normalized serum TSH, not all systemic markers of thyroid hormone signaling were normalized, including serum LDL and total cholesterol. The failure to restore a euthyroid state with levothyroxine monotherapy may explain the ∼15% dissatisfaction with hypothyroid patients treated with T_4 alone. This is supported by Peterson et al. (17) who conducted an online survey of hypothyroid patients to determine their level of satisfaction with their current therapy or their physician. Higher satisfaction was reported by patients receiving desiccated thyroid extract followed by patients receiving combination T_4 plus T_3. The lowest satisfaction level was found in patients on T_4 monotherapy. The study does not distinguish between physician and therapy dissatisfaction, but one can assume they are related.

A recent study reported on quality of life measures in hypothyroid patients using the Thyroid Patient-Reported Outcome (ThyPRO-39) questionnaire. Patients were on T_4 monotherapy but experiencing persistent symptoms (18). Patients switched to combination LT_4/LT_3 combination therapy showed an improvement in quality of life measures, which was not associated with a change in TSH.

In preclinical studies, Escobar-Morreale et al. (19) demonstrated that the infusion of T_4 alone to hypothyroid rats, at any dose, cannot normalize TSH, T_4, and T_3 in the blood, or in all tissues of the hypothyroid animal. In order to ensure normal T_3 levels in all tissues supraphysiological plasma levels of T_4 resulted. The minimal dose of T_4 that resulted in normal plasma T_4 and T_3 was insufficient to normalize the concentration of T_3 in most tissues analyzed including heart, lung, liver and kidney. Results also demonstrated differential uptake of circulating T_4. This implies that in humans current replacement therapy with T_4 alone would not be adequate to render all tissues euthyroid. In a second study by the same group (20) infusion of hypothyroid rats with T_4 alone or T_4 in combination with T_3 (at three different doses) demonstrated that tissue euthyroidism is only possible when T_4 is infused together with T_3. In all treatment groups, plasma T_4 was normalized but TSH and T_3 in plasma and T_3 in most tissues were only normalized with the combination of T_4 plus T_3.

In a clinical study by Celi et al. (21) 14 hypothyroid patients received either T_4 monotherapy or T_3 administered three times a day at doses that produced equivalent normalization of serum TSH. No difference was noted in TSH between groups. Results demonstrated that T_3 treatment resulted in significant weight loss and a more favorable lipid profile (decreased total cholesterol and LDL cholesterol) when compared to T_4 treatment, implying adequate tissue levels of serum T_3 in these patients. T_3 treatment produced no differences in cardiovascular function (heart rate, blood pressure, or exercise tolerance), HDLs, or in insulin sensitivity when compared to T_4.

An easy to use hypothyroid symptom scale that identifies those patients with persistent symptoms is a critical component for addressing the needs of the 15% of patients who are not "satisfied" on their current therapy. In the case of suspected inadequate thyroid hormone replacement, the clinician may opt for LT4 dose increase, change in LT4 formulation or the addition of liothyronine (22). The proposed hypothyroid scale correctly identified the subjects with TSH elevation and clinical/subclinical hypothyroidism based on their clinical signs and symptoms. In this particular subset of patients, the hypothyroid symptom scale showed a statistically significant improvement in the sum of the signs and symptoms with the normalization of the subjects' thyroid function. At the same time, the sum of the signs and symptoms of patients with no change in medication remained unchanged, suggesting a reproducibility of this hypothyroid scale. This scale provides the clinician with an easily applied clinical tool to assess the adequacy of treatment in hypothyroid patients.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

Reviewed by the North-Shore Hospital IRB in Manhasset, NY. Study was found to be EXEMPT as no identifying information was obtained.

AUTHOR CONTRIBUTIONS

MB and IK: data collection, analysis, and manuscript preparation. SD: manuscript preparation and data analysis.

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Functional and Symptomatic Individuality in the Response to Levothyroxine Treatment

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Background: For significant numbers of patients dissatisfied on standard levothyroxine (LT4) treatment for hypothyroidism, patient-specific responses to T4 could play a significant role.

Aim: To assess response heterogeneity to LT4 treatment, identifying confounders and hidden clusters within a patient panel, we performed a secondary analysis using data from a prospective cross-sectional and retrospective longitudinal study.

Methods: Multivariate and multivariable linear models adjusted for covariates (gender, age, and BMI) were stratified by disease-specific treatment indication. During follow-up, pooled observations were compared from the same patient presenting either with or without self-reported symptoms. Statistical analysis was extended to multilevel models to derive intra-class correlation coefficients and reliability measures during follow-up.

Results: Equilibria between TSH, FT4, and FT3 serum concentrations in 342 patients were examined by treatment indication (benign goiter, autoimmune thyroiditis, thyroid carcinoma), consequently displaying complex interactive response patterns. Seventy-seven patients treated with LT4 and monitored for thyroid carcinoma presented, in association with changes in LT4 dose, either with hypothyroid symptoms or symptom-free. Significant biochemical differences appeared between the different presentations. Leveled trajectories by subject to relief from hypothyroid symptoms differed significantly, indicating distinct responses, and denying a single shared outcome. These were formally defined by a high coefficient of the intraclass correlation (ICC1, exceeding 0.60 in all thyroid parameters) during follow-up on multiple visits at the same LT4 dose, when lacking symptoms. The intra-personal clusters were clearly differentiated from random variability by random group resampling. Symptomatic change in these patients was strongly associated with serum FT3, but not with FT4 or TSH concentrations. In 25 patients transitioning from asymptomatic to symptomatically hyperthyroid, FT3 concentrations remained within the reference limits, whilst at the same time marked biochemical differences were apparent between the presentations.

Conclusions: Considerable intra-individual clustering occurred in the biochemical and symptomatic responses to LT4 treatment, implying statistically multileveled response

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groups. Unmasking individual differences in the averaged treatment response hereby highlights clinically distinguishable subgroups within an indiscriminate patient panel. This, through well-designed larger clinical trials will better target the different therapeutic needs of individual patients.

Keywords: intra-class correlation, response heterogeneity, LT4 treatment, thyroid carcinoma, thyroid homeostasis, setpoint, ergodicity

INTRODUCTION

Despite lacking the minor component of triiodothyronine (T3) physiologically co-secreted with thyroxine (T4) by the healthy human thyroid gland, in thyroid failure monotherapy by levothyroxine (LT4) replacement remains the standard treatment for patients with primary hypothyroidism (1, 2). LT4 is one of the most frequently prescribed drugs with a long history of successful use and favorable safety record (3–5). Administered in variable doses, dose adequacy for a hypothyroid patient is determined by biochemically defined treatment targets based mainly on TSH measurements (2). This marks a historical shift from earlier regimens primarily aiming at symptom relief (6). However, despite achieving appropriate biochemical treatment targets with LT4, as defined by current guidelines (2), a substantial fraction of patients continues to report persisting symptomatology expressing their dissatisfaction with the standard treatment (7). The magnitude of the problem has recently been re-emphasized by a large online survey conducted by the American Thyroid Association where satisfaction with LT4 treatment reported by the 12,146 respondents was only at median 5 on a scale of 1–10 (8). A prospective study by Winther et al. using a validated thyroid specific QoL questionnaire and following hypothyroid patients with autoimmune thyroiditis, concluded that QoL outcome measures improved but a full recovery was not achieved after 6 months of treatment with LT4 (9). While patients and doctors reported some success with the addition of T3 and guidelines by the European Thyroid Association acknowledge a potential benefit of T3/T4 combinations to some patients this subject overall remains contentious (7, 10–12).

Variable patient experiences with LT4 treatment and the possible existence of differently responding subgroups of patients have long been suspected (13), but formal analysis of this problem with robust statistical methods is seriously lacking. A biochemical dissociation in the equilibria or so called setpoint between TSH, FT4, and FT3 has been increasingly recognized, both in untreated subjects and in patients treated with LT4 (5, 13). Patients on LT4 display considerable variation in their biochemical and symptomatic treatment response, along with the manifestation of a pronounced disjoint between FT3 and TSH concentrations, compared to the relationship in thyroid health (14–16). This may also pertain to such intrinsic differences in patient response as to encourage an exploration of risk stratification. In this respect, the Rotterdam study documented an increased risk of both atrial fibrillation and sudden cardiac death in an untreated euthyroid population with higher LT4 serum concentrations within its reference range, yet uncorrelated with TSH concentrations (17, 18).

In the present study, we question to what extent the response to LT4 treatment, as expressed in the respective equilibria between the thyroid parameters TSH, FT4, and FT3, may differ between athyreotic patients with thyroid carcinoma and benign entities such as autoimmune thyroiditis or goiter post-surgery. In a panel of athyreotic patients with thyroid carcinoma followed long term on LT4 replacement, we assessed the biochemical alterations in individual subjects with symptoms before and after symptom relief. We were particularly interested in possible implications of ergodicity arising during long-term follow-up from a narrow intra-individual variation of thyroid hormones.

METHODS

Patients

Data for this secondary analysis were collected as part of two previously reported trials, a cross-sectional prospective trial and a retrospective longitudinal study (19–21). The prospective trial was registered (www.ClinicalTrials.gov, NCT 01969552), ethically approved, and all participants gave written informed consent, and the retrospective study was approved by the local authorities in data protection. Both studies were conducted in an outpatient setting, prospectively from 2013 to 2014 in 1912 patients with various thyroid diseases and retrospectively from 2008 to 2016 in 319 patients with thyroid carcinoma routinely monitored at 2,309 visits (19, 21). Only LT4-treated out-patients without known comorbidity were included in the present study, and we also included only visits after hypothyroidism was biochemically controlled as defined by both a TSH < 4 mIU/l and FT4 > 10 pmol/l, while FT3 concentrations were within the reference limits. All measurements were obtained on unchanged stable medication in equilibrium. Indication for LT4 treatment resulted from three different indications, namely benign goiter, primary hypothyroidism due to thyroid autoimmune disease as evidenced by the presence of peroxidase antibodies (TPO Ab), and total thyroidectomy due to thyroid carcinoma. Patients with thyroid carcinoma were regularly monitored at 6 month intervals for the first 5 years after thyroidectomy and 12 month intervals thereafter in tumor-free patients, and followed long-term. Patient characteristics are tabulated as relevant for this study (**Tables 1**, **2**).

Details were collected on patient history and medication, demographic factors (gender, age, BMI), physical examination, ultrasound, and laboratory tests (FT3, FT4, TSH, TPO Ab, and TSH-receptor antibodies (TSH R Ab) in TPO Ab positive cases only). In the longitudinal study only, any patient complaints, specific and non-specific, were freely communicated during visits

TABLE 1 | Patient characteristics in the cross-sectional study.

Parameter	Goiter	Autoimmune thyroiditis	Thyroid carcinoma	*P*-value*
Patients (*n*)	111	95	136	–
Gender (female/male)	83/17%	92/8%	71/29%	<0.001
Age (years)	59.5 (12.1)	51.9 (15.8)	54.6 (14.1)	<0.001
BMI (kg/m^2)	26.8 [24.2, 30.8]	27.2 [23.5, 29.7]	28.3 [24.8, 33.0]	<0.001
Weight adjusted LT4 dose (μg/kg BW /day)	1.11 [0.88, 1.47]	1.27 [0.95, 1.67]	1.69 [1.52, 2.01]	<0.001
FT3 (pmol/l)	4.76 (0.52)	4.62 (0.56)	5.09 (0.72)	<0.001
FT4 (pmol/l)	17.15 [15.9, 19.6]	17.0 [15.1, 18.9]	20.4 [18.6, 22.8]	<0.001
TSH (mIU/l)	0.81 [0.42, 1.27]	1.32 [0.57, 2.04]	0.17 [0.03, 0.88]	<0.001

**P-values were derived by ANOVA or, in case of non-normally distributed parameters, Kruskal-Wallis test.*

TABLE 2 | Characteristics of patients with thyroid carcinoma in the longitudinal study.

Parameter	Median [interquartile range]
Patients (*n*)	319
Visits (*n*)	2,309
Follow-up duration (months)	63 [46, 81]
Follow-up intervals (months)	Six over the first 5 years, 12 thereafter, if tumor-free
Gender (female/male)	72/28%
Age at initial presentation (years)	50.1 [41.1, 62.0]
Body mass index (kg/m^2)	28.2 [24.3, 31.3]
Tumor type	Papillary 69%, follicular 19%, other 12%
Tumor stage at initial presentation	pT1 46%, pT2 20%, pT3 12%, pT4 3%, N1 12%, M1 4%
Ablative treatment	surgery 100%, plus radioiodine 92.5%
Weight adjusted LT4 dose (μg/kg BW/day)	1.84 [1.62, 2.14]
TSH (mIU/l)	0.07 [0.01, 0.46]
FT3 (pmol/l)	5.15 [4.60, 5.80]
FT4 (pmol/l)	22.3 [19.6, 25.4]

in an open format, avoiding suggestive or standardized questions, and documented as such. The documented complaints were later independently categorized by a specialist into thyroid-unrelated symptoms (e.g., back pain), hypothyroid symptoms (e.g., tiredness, fatigue, lack of energy, cold intolerance, weight gain) and hyperthyroid symptoms (e.g., nervousness, irritability, restlessness, anxiety, rapid pulse, palpitations, trembling, heat intolerance, unwanted weight loss). The terms complaint and symptom are used synonymously here. During follow-up adjustments were made to the LT4 dose, either in response to individual patient complaints or according to the general treatment strategy including the individual risk profile and changes in guideline recommendations over the years (21).

Thyroid Ultrasound

In all subjects, thyroid volume, echo-density, and nodularity were examined by ultrasound (10 MHz transducer). Thyroid volume was determined by the ellipsoid formula (longitudinal diameter × width × depth × 0.5 cm^3) and summation of lobe volumes. Reference values are <18 ml for female and <25 ml for male subjects.

Laboratory Methods

TSH was measured with an automated direct chemiluminescence method (TSH3-Ultra ADVIA Centaur XP, Siemens Healthcare Diagnostics, Erlangen, Germany). The standard curve was calibrated with the 3rd WHO Standard for hTSH (IRP 81/565). Functional sensitivity was 0.008 mIU/l, intra-assay variation 1.4–2.4%, and inter-assay imprecision 0.9–2.9%. FT3 and FT4 were measured on the same platform, showing intra-assay CVs from 2.4 to 3.1% or 2.2 to 3.3% and inter-assay CVs from 2.3 to 3.9% or 2.5 to 4.0%, respectively. Assay performance characteristics have been reported (22). Laboratory-evaluated reference intervals were as follows, 0.4–4 mIU/l for TSH, 3.1–6.8 pmol/l for FT3, 10–23 pmol/l for FT4.

TPO Abs were measured with a competitive chemiluminescence method (ADVIA Centaur XP, Siemens Healthcare Diagnostics, Erlangen, Germany) and TSH-R Abs with an ELISA (EUROIMMUN AG, Lübeck, Germany). Reference ranges were for TPO Ab <60 IU/ml and for TSH-R Ab <2 IU/l.

Statistical Methods

Descriptive data are shown as mean (standard deviation, SD) or median (interquartile range, IQR). Non-normally distributed TSH values were natural logarithmically transformed. Between-two-group comparisons for continuous variables were based on Welch's *t*-test or, if normality could not be assumed, Wilcoxon's rank-sum test. More than three independent groups were compared using ANOVA or a Kruskal-Wallis test. Chi-squared test with Yates' correction for continuity was

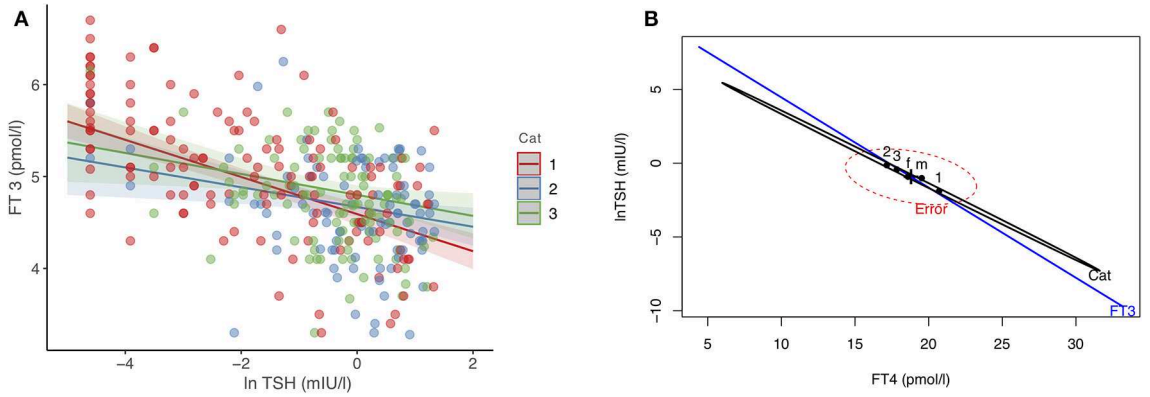

FIGURE 1 | (A,B) Effect plots of the complex interdependency between FT3, FT4, and TSH. **(A)** shows the relationship between FT3 and TSH, examined by treatment category, after adjusting for FT4 concentrations, gender, age, and BMI. **(B)** The resulting equilibria between FT4 and TSH (as surrogate markers for setpoints), associated with treatment category and FT3 concentrations in a significant interaction (Pillai-test $p < 0.001$, see Results for details). The three treatment categories refer to patients with thyroid carcinoma (1), autoimmune thyroiditis (2), and benign goiter (3). f indicates female, and m male gender. Cat, disease category. Regression lines are surrounded by the 95% confidence limit.

used for categorical variables. Pooled observations at either symptomatic or asymptomatic presentations derived from the same patient were compared using a paired *t*-test or the signed rank Wilcoxon test. Multivariable and multivariate linear models, adjusted for disease entity, gender, age, and BMI were used to assess associations across subjects between thyroid parameters including–when significant–more complex multiplicative interactions between them. MANOVA tests for the multivariate models relied on Pillai's test statistic. Residual plots were inspected to verify model assumptions. Changes during follow-up in the binary outcomes for the presence or absence of symptoms and continuous thyroid parameters were assessed using generalized linear mixed models with a restricted maximum likelihood estimator (REML) and a binomial or Gaussian link function, respectively, appropriately accounting for within-variation and intra-subject correlations for repeated measurements per subject in the longitudinal design (23). Effect plots predict the binary outcome as a probability response on a linearized logit scale or the natural response of a continuous outcome. Relative risks (RR) are reported in Results. Model performance was compared by both *F*-test and Akkaike's information criteria (AIC). These models were formulated as unconditional means models to derive estimates on intra-class correlation coefficients (ICC1) and reliability (ICC2) for thyroid hormones obtained at multiple occasions under stable conditions during follow-up. Random group resampling was performed to differentiate personal group-level properties from random group variability (24, 25). Power simulations were done according to the methods described and implemented by Bliese (24, 25). All tests were two-sided with $p < 0.05$ denoting statistical significance. Variables were considered explanatory without adjusting for multiple comparisons. We used the R statistical software environment (version 3.5.2 for Mac) (26) with the added packages lme4 1.1-19 (23), effects 4.1-0 (27), heplots 1.3.-5 (28), sjstats 0.17-3 (29), and multilevel 2.6 (24, 25).

RESULTS

Patient characteristics of LT4-treated patients are summarized in **Table 1** for the cross-sectional study and in **Table 2** for the longitudinal study.

Cross-Sectional Study

In 342 patients treated with LT4, we examined their equilibria and biochemical response heterogeneity by disease entity, benign goiter ($n = 111$), autoimmune thyroiditis ($n = 95$), thyroid carcinoma after thyroidectomy ($n = 136$). Using FT3 levels achieved as dependent outcome in a multivariable linear model, disease category (significantly steeper relationship in the carcinoma group, $p = 0.02$), FT4 (0.03 pmol/l per pmol/l, 95% CI [0.014, 0.052], $p < 0.001$), and TSH (lnTSH −0.17 pmol/l per mIU/l, 95% CI [−0.21, −0.13], $p < 0.001$) concentrations were all significantly independent predictors. The influence of the other adjusted covariates present in the model was as follows, gender (0.42 pmol/l higher for men, 95% CI [0.29, 0.56], $p < 0.001$), age (−0.012 pmol/l per year 95% CI [−0.015, −0.008], $p < 0.001$), BMI (−0.002 pmol/l per kg/m^2, 95% CI [−0.011, 0.006], $p = 0.60$). **Figure 1A** shows the TSH-dependent and FT4-adjusted FT3 response by treatment category. Conversely, in a multivariate model, FT3 concentrations interacted with the treatment category in predicting the combined outcomes for FT4 and TSH, used as surrogates for setpoints. This interaction was highly significant (Pillai test, $p < 0.001$), and remained so after adjusting for gender, age and BMI (Pillai test, $p < 0.001$). **Figure 1B** shows the derived trajectories for the relationships and estimates the resulting FT3-dependent equilibria (setpoints) between FT4

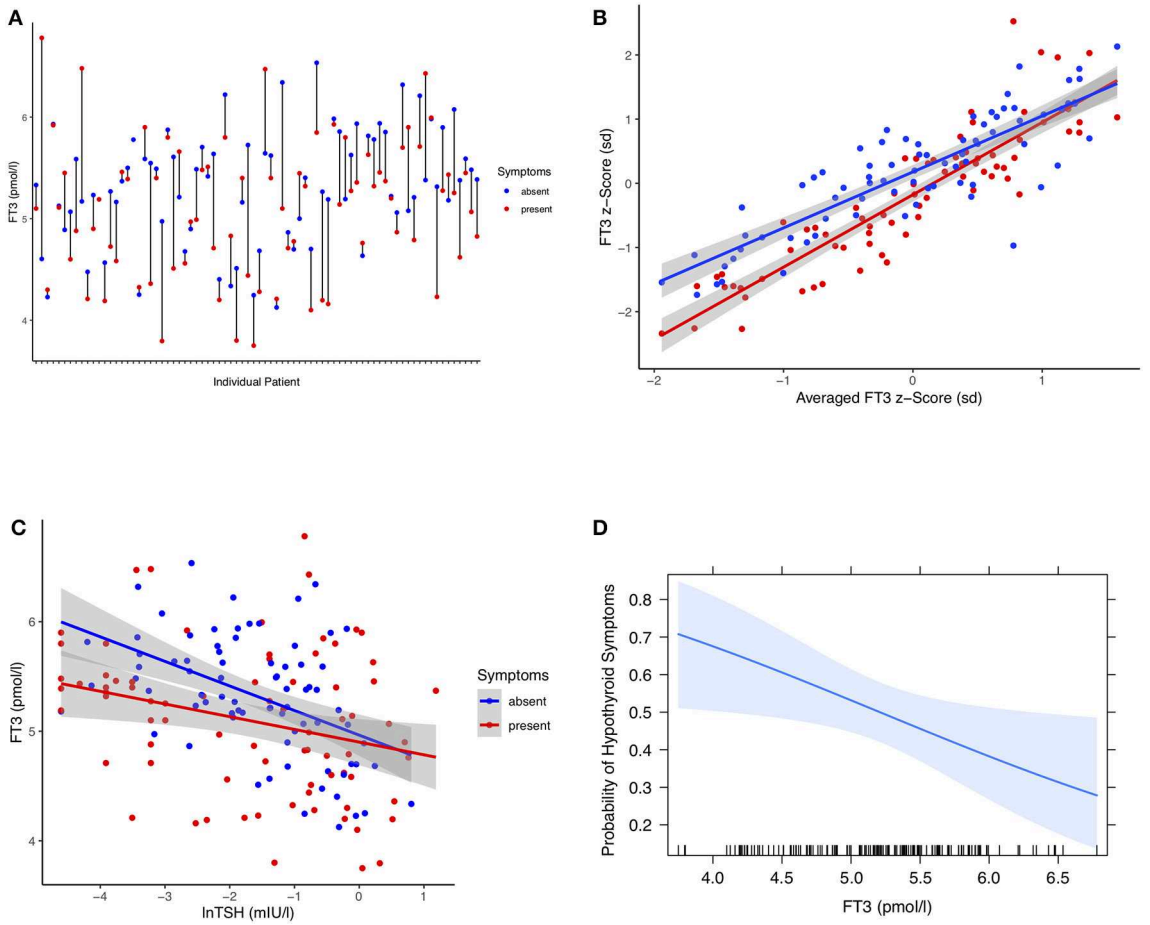

FIGURE 2 | (A) Difference plot of serum FT3 concentrations in 77 individual patients at either visits with hypothyroid symptoms or asymptomatic presentations. Each point refers to pooled measurements obtained from a single patient during follow-up and averaged over multiple either symptomatic or asymptomatic visits after adjusting LT4 dose (see Results). **(B)** FT3 z-scores of either symptomatic or asymptomatic presentations plotted against the averaged scores over all visits per patient. FT3 concentrations are mean-centered and units are expressed in standard deviations. This shows that the corrective FT3 difference associated with relief of self-reported hypothyroid symptoms increased progressively with increasing distance from the center. Shaded areas indicate the 95% confidence limit for the fitted regression line. **(C)** FT3 change relative to TSH stratified by symptomatic and asymptomatic presentations of the same patients. The intersecting points did not move along a shared trajectory between the two conditions but were significantly shifted, progressively so toward lower TSH concentrations (see Results). **(D)** Probability of hypothyroid symptoms as a function of circulating FT3 concentrations. The probability of the presence of hypothyroid symptoms at a given FT3 level for these patients was derived by a multilevel model accounting for intra-class and between-subject variation (see Methods and Results). The shaded area indicates the 95% confidence limit of the probability curve. The vertical ticks on the x axis indicate the observed individual values.

and TSH serum concentrations by treatment category and FT3 levels.

Longitudinal Study

In a longitudinal series of 319 patients with thyroid carcinoma followed at 2,309 visits for 63 months (median, IQR 46, 81), we assessed the individual treatment responses. Of particular interest were 77 patients with changing hypothyroid symptomatology following LT4 dose adjustment during follow-up. The statistical comparison between pooled paired observations, averaged over multiple visits (median 9, IQR 6, 12) at either symptomatically hypothyroid or asymptomatic presentations, was as follows, weight-adjusted LT4 dose (−0.081 μg/kg BW, 95% CI [−0.14, −0.024], paired *t*-test: $p = 0.006$), TSH concentrations (0.14 mIU/l [−0.03, 0.31], paired signed Wilcoxon test: $p = 0.47$), FT4 concentrations (−1.05 pmol/l, 95% CI [−1.83, −0.28], paired *t*-test: $p = 0.009$), and FT3 levels (−0.22 pmol/l, 95% CI [−0.36, −0.08], paired *t*-test: $p = 0.002$), all except TSH being significantly lower at presentations with hypothyroid symptoms. A difference plot of FT3 measurements between the symptomatic and asymptomatic pairs revealed considerable diversity among individual patients in their start and end levels and respective distances between the two levels (**Figure 2A**). After mean-standardizing the FT3 concentrations, we plotted the z-scores for FT3 of either symptomatic or asymptomatic presentations against the average scores over all visits per patient (**Figure 2B**). This shows that the corrective effect size required for relief of hypothyroid symptoms increased with the distance from the

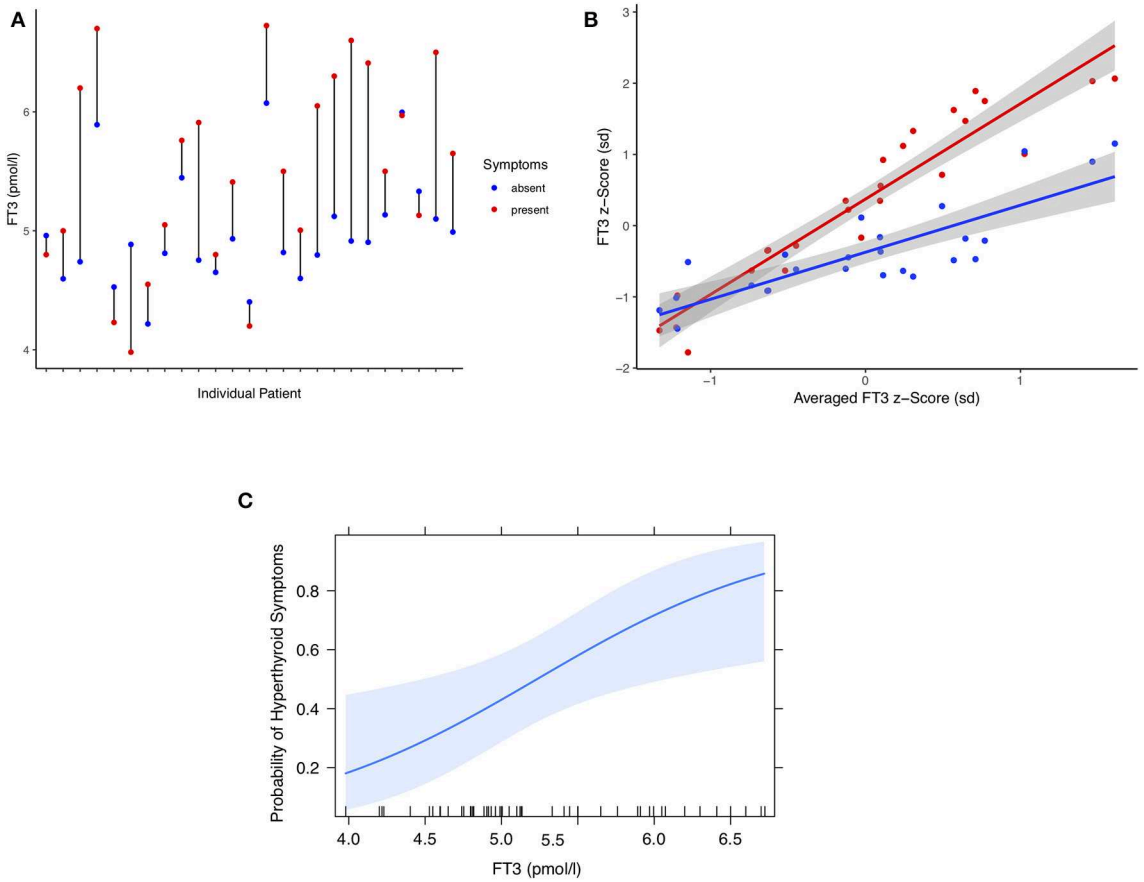

FIGURE 3 | (A) Difference plot of serum FT3 concentrations in 25 individual patients at either hyperthyroid or symptom-free presentations. Each point refers to the pooled measurements over multiple either symptomatic or asymptomatic visits (see Results). **(B)** FT3 z-scores of either symptomatic or asymptomatic presentations plotted against the averaged scores over all visits per patient. Shown are mean-centered standardized FT3 concentrations and a fitted regression line surrounded by its 95% confidence limit (shaded area). **(C)** Probability of hyperthyroid symptoms in these patients as a function of circulating FT3 concentrations. A multilevel model was used to estimate the probability (see Methods and Results). The shaded area indicates the 95% confidence limit of the fitted curve. The vertical ticks on the x axis indicate the observed individual values.

center. Looking at FT3 change relative to TSH, symptomatic and asymptomatic observations did not move along a shared trajectory but were significantly shifted (0.23 pmol/l 95% CI [0.36, 0.11], $p < 0.001$) (**Figure 2C**). On average, the rate of hypothyroid symptoms increased with lower FT3 concentrations (**Figure 2D**). FT3 serum concentrations (RR 0.70 per pmol/l, 95% CI [0.49, 0.96], $p = 0.03$), but not FT4 concentrations (RR 0.95 per pmol/l, 95% CI [0.91, 1.00], $p = 0.053$), and TSH concentrations (lnTSH RR 0.98 per mIU/l, 95% CI [0.87, 1.09], $p = 0.73$) were significantly predictive of the presence of hypothyroid symptoms in these patients. In this respect, a combination of all three covariates FT3, FT4, and TSH was not more informative, compared to FT3 measurements alone (F-test $p = 0.14$, AIC difference 0.11). Confounders in the cross-sectional study, namely gender ($p = 0.30$), age ($p = 0.57$), and BMI ($p = 0.93$), were non-influential but adjusting for these covariates slightly reduced the variation of the significant FT3 influence (adjusted RR 0.55, 95% CI [0.31, 0.87], $p = 0.008$).

Similar to the hypothyroid complaints, the pooled observations from 25 individual patients were compared when they were either symptom-free or presented with hyperthyroid symptoms (**Figures 3A–C**). Differences were highly significant for weight-adjusted LT4 dose (0.23 μg/kg BW, 95% CI [0.12, 0.35], paired t-test: $p < 0.001$), TSH concentrations (−0.44 mIU/l [−0.60, −0.29], paired signed Wilcoxon test: $p < 0.001$), FT4 concentrations (4.20 pmol/l, 95% CI [2.83, 5.58], paired t-test: $p < 0.001$), and FT3 levels (0.53 pmol/l, 95% CI [0.26, 0.80], paired t-test: $p < 0.001$). For individuals, FT3 and symptomatic change are shown in **Figure 3A**. Standardized effect sizes are depicted in **Figure 3B**. The probability of hyperthyroid symptoms increased with higher serum concentrations of FT3, as shown in **Figure 3C**. Relative risk estimates with increasing concentrations were as follows, FT3 1.54 per pmol, 95% CI [1.13, 1.79], $p < 0.001$), FT4 1.17 per pmol, 95% CI [1.06, 1.28], $p = 0.003$), and lnTSH 0.42 per mIU/l, 95% CI [0.24, 0.70], $p < 0.001$).

Intra-Class Correlations and Reliability

We estimated the intra-class correlation coefficient (ICC1) and reliability (ICC2) over the follow-up period in 141 patients at

435 visits on a stable unchanged LT4 dose of 125 μg/day and in the absence of symptoms. The individual patients displayed a high intraclass correlation for all thyroid parameters, FT3 0.61, FT4 0.67, and TSH 0.67. FT3-dependent multilevel trajectories to relief of hypothyroid symptoms also proved highly individually variable, ICC1 0.64.

All parameters showed excellent group-mean reliability FT3 0.83, FT4 0.86, TSH 0.86, indicating that the individuals do not form random groups and can be reliably differentiated. To estimate how much appropriately accounting for level properties may reduce variance or, conversely, if ignored, inflate variance we compared the variance of randomly resampled pseudo-groups with the real variance in the actual groups where every patient formed their own group during follow-up. This demonstrates a highly significant ($p < 0.001$) and pronounced influence of personal grouping, as opposed to random grouping, the mean within-group variance for the random FT3 sample being 0.46, compared to 0.26 for the real data for FT3.

Potential Bias of Ignoring Intra-Class Correlations

A bias may arise, for instance in an RCT, if the individual levels are disregarded and the data is treated as though it was independent. Simulating a hypothetical design with two groups, 40 subjects per group, moderate between-variable correlation ($r = 0.47$) and intra-class correlation of 0.61 for the outcome variable, the *t*-test-based power estimate of such a trial would be reduced from 92 to 48% if the analysis fails to account for the observed multilevel structure in the data. A sufficiently powered (93%) larger trial with a group size of 200 subjects and a lower correlation of 0.25 under otherwise identical conditions would become underpowered by ignoring the level properties in the sample (power estimate 63%). Increasing group size to 500 subjects in the case of three groups and two weakly correlated variables ($r = 0.10$) would not remedy the lack of power caused by averaging (54%), compared to leveling (91%) the outcome.

DISCUSSION

Although in health a narrow intra-individual variation of thyroid hormones has long been recognized (30), its application to the treatment of patients with LT4 has not been rigorously examined. This study has uncovered considerable inter-individual variability and intra-class correlations in the biochemical and symptomatic responses to LT4 treatment within a patient panel. For instance, failure to account for a multilevel structure in the data, if present in a randomized controlled clinical trial (RCT), may mask potential treatment effects. This will result in the reduction of statistical power when predicting treatment-associated outcomes in hypothyroid patients on LT4.

Non-ergodicity of Thyroid Parameters

In probability theory, the definition of an ergodic dynamical system is that it displays the same behavior when averaged over time as averaged over the space of all the system's states in its phase space (31). The mathematical definition emphasizes that for group membership all group members must share the same moments, namely means, variances and covariances (32). Ergodicity is a requirement when generalizing from the population to the individual level (32–35). Because the implicitly assumed ergodicity of thyroid parameters does not hold true, thyroid reference ranges are fundamentally inappropriate and should be replaced by personal setpoints (5). Ergodicity is most particularly challenged in situations where both trait-like differences exist among individuals and structural change occurs over time. In thyroid patients, the concept relates to trait-like personal setpoints (equilibria between TSH and FT4, FT3) that undergo structural change during follow-up. The determination of intra-class correlations (ICC) provides a quantitative measure on influences associated with either a subject or a particular situation.

Intra-Class Correlation of Thyroid Parameters

In our longitudinal series, we documented substantial intra-class correlation for all thyroid parameters. This characterizes the thyroid status largely as a personal trait, which varies under stable medication more between subjects, and less so within a person on different occasions. Random variability was ruled out by a permutation test, confirming both the multi-level properties and individual heterogeneity among the patients within the panel. Whenever the intra-class correlation coefficient is found to be large, we cannot confidently use aggregated statistical methods on these data that assume independence, because estimates of variance, and therefore *p*-values, become insufficiently robust. As pointed out by Fisher et al., best-practice guidelines derived from RCTs in such conditions tend to overestimate the accuracy of aggregated statistical estimates (35). Ecological fallacy, collider stratification bias and Simpson's paradox are variations of the problem with serious implications, as exemplified by the market retraction of an approved drug (34–38).

Setpoint theory may explain the high degree of individuality physiologically observed in thyroid parameters (39, 40). In the thyroid healthy state, the so-called setpoint delivers a homeostatically defined multivariate expression of the stable equilibrium between interlocked pairs of TSH and FT4 (41). The resulting distribution of clustered setpoints is fundamentally different from the process of using univariate reference ranges for TSH or FT4, rather requiring multivariate and multileveled approaches (5, 41). In the event of a disease or under the influence of LT4 treatment, personal set points may be conditionally redistributed (13, 14). Consequently, the TSH level previously appropriate for thyroid health cannot equally serve as a treatment target in the same person (42).

From an evolutionary point of view, moderate diversity in their personal setpoints and response heterogeneity among individuals within a human or animal population makes sense, attenuating excessive reactions, and abrupt transitions to changing environmental conditions in a community (43–45).

Intra-Class Correlation and Patient Complaints

The reasons for persisting patient complaints are not well-understood and thyroid-related symptoms may overlap with a plethora of non-specific complaints (8, 46–60). Also, drugs containing LT4 or LT3 may display mild antidepressant pharmacological properties (61). In an ergodic framework, we may question whether complaints relate to the dynamic structure of the underlying thyroid process or are independent "traits" of the individual and derive quantitative estimates for the two components. Concerns about inexplicable variation (62, 63) should be advanced from the descriptive level to analytical study. A focus on idiographic patterns following individual patients receiving LT4 long term on multiple occasions, as in this retrospective study, limits the impact of inter-personal variation. It reduces thereby the importance of chief non-thyroidal confounders of cross-sectional studies such as gender, age and BMI as well as treatment-related variation in the biochemical equilibria across different disease entities, such as thyroid carcinoma, autoimmune thyroiditis, and goiter. This may uncover subtle differences that may otherwise remain hidden within a noisy background and go undetected by statistically inappropriate averaging.

Strengths and Limitations of the Study

The present study is one of the first of its kind in conducting an intra-class correlation analysis in the treatment response to LT4 over multiple presentations in a large sample of patients with thyroid carcinoma followed for several years. It has however several limitations. This is a secondary analysis; the primary clinical study outcomes of both the prospective cross-sectional trial and retrospective longitudinal study have previously been reported (19, 21). Although patients with known comorbidities, interfering comedication, and clinical conditions in which elevated TSH levels persisted (e.g., non-adherence, LT4 malabsorption) were excluded from this analysis remaining subclinical pathologies are part of the biological variation (64). The present analysis focusses on the framework of ergodicity and multileveled patterns of the responses to LT4 treatment within a patient panel. Although the study design was uncontrolled, the findings of the ICC analysis are pertinent to prospective studies and RCTs and may aid in improving future trials. As hypothyroid symptoms inherently overlap with non-specific or hyperthyroid complaints, a few misclassifications are inevitable but are of little apparent influence on the main tendencies for each symptom category (**Figures 2**, **3**). More importantly, patient expectancy remains a bias that has not been robustly addressed in any thyroid trial including RCTs (65). The American Food and Drug Administration (FDA) therefore demands drugs to be evaluated under "actual conditions of use"–a requirement met by none of the many RCTs on QoL outcomes for LT4 and T4 T3 combinations (10, 11, 65).

Symptom Evaluation

Subjective symptoms as experienced by the patient were freely communicated during routine visits in an open format, being retrospectively and independently categorized into hypothyroid, hyperthyroid, or thyroid-unrelated complaints. While unstandardized, this process avoids any suggestive interrogation. The presence of leading symptoms may successfully substitute for the use of more complex QoL questionnaires, and instantaneous assessment offers higher precision and sensitivity to change, compared to retrospective ratings (66, 67). Due to their non-ergodic behavior TSH and thyroid hormone levels associated with the presence or absence of hypothyroid symptoms considerably overlap among individual patients. Individual trajectories to symptom relief start from different levels and end at different targets. Intra-class clustering and shifts in the treatment response reduce the discriminatory power of averaged between-subject comparisons in trials, including RCTs, as demonstrated in Results.

T4 mainly acts as a circulating pro-hormone, requiring both prior transmembrane transport and enzymatic activation to exert a multitude of genomic actions through nuclear thyroid hormone receptor binding (68–71). Both T3 conversion rates and thyroidal T3 secretion are subject to central control by TSH (72–76). The loss of functioning thyroid tissue and/or the TSH-lowering effect of LT4 treatment may impair this compensatory mechanism (16, 19, 77). Due to the expression of distributional individuality and subsequent disruption of the TSH-FT4 correlation, subclinical hypothyroidism is an indeterminate and unreliable disease classifier (5, 13). This dissolves the existing relationships in an individual prior to thyroidectomy, re-adjusting the setpoint and re-setting the equilibria between TSH, FT4, and FT3 in thyroid disease, compared to the healthy state (14, 15, 42, 78). While regarded as essential in maintaining narrow individual serum concentrations of the respective hormones (30), the non-ergodic behavior has yet to be transferred to the treatment situation where population range-based recommendations are paramount in guidelines (2).

Clinical Implications

Dissimilar clusters or individuals may have conditional requirements for optimum treatment success different from the averaged population and risk profiles may also be shifted. This is in accord with a recent prospective study defining the optimum TSH target slightly below the lower reference limit for patients with thyroid carcinoma treated with LT4, based on the examination of surrogate markers (79). TSH-independent risk profiles have also been demonstrated by the Rotterdam study in euthyroid subjects, although this study did not include a sufficient number of LT4-treated subjects (17, 18). We note that in our study patients with uncontrolled or refractory hypothyroidism (TSH $>$ 4 mIU/l) were excluded and TSH suppression was primarily motivated by tumor control–which is now managed differently–not symptom control (80–83). We do not infer that patients should have a suppressed TSH, rather that the personal levels expressed in the treated condition have different meanings, compared to the untreated situation. Neither does a TSH measurement within its reference limits guarantee that a patient will be symptom-free, nor that a presumably healthy person by this definition may not suffer serious adverse consequences such as atrial fibrillation (17). We and others proposed a more personal definition of "euthyroid," based on individual traits

(setpoints) and dynamic changes between the relationships of all three thyroid parameters TSH, FT4, and FT3 (5). This extends to both genetically determined fingerprints and treatment-related alterations in the expression of personal setpoints and includes other allostatic expressions of individuality (e.g., in their gut microbiome) affecting iodothyronine homeostasis (84–90).

Dissimilarities in the treatment responses between individual patients are particularly apparent when patients on LT4 display substantial variation in their T4–T3 conversion efficiency and pronounced disjoints between their serum TSH and FT3 concentrations (16, 77). Importantly, FT3 levels relative to TSH in symptomatic vs. asymptomatic presentations of the same patients did not move along a shared trajectory but were shifted upward when the patients transitioned from the symptomatically hypothyroid to the asymptomatic condition. Depending on the patient presentation, the addition of LT3 is increasingly considered by thyroid experts worldwide (12). Differential treatment of identifiable dissimilar subpopulations, e.g., with persistently low FT3 concentrations despite normalized TSH (77), appears feasible, but was not tested in this study and awaits further proof.

SUMMARY

Complex patterns emerge between TSH, FT4, and FT3 in patients treated with LT4 during follow-up in response to the treatment and changes in LT4 dose, displaying a high degree of intra-class correlation and multileveled structure. This invokes the danger of inappropriate statistical averaging in clinical trials, mandating a stronger focus on within-subject analyses according to ergodic principles. It emphasizes a need to better define personal treatment outcomes and individual risk profiles in patients receiving LT4 alone or, similarly, a combination of T3 and T4.

ETHICS STATEMENT

The prospective trial was registered (www.ClinicalTrials.gov, NCT 01969552) and the protocol was ethically approved by the Ethical Committee of the University of Münster, Germany. All participants gave written informed consent. The retrospective analysis was specifically approved by the local authorities in data protection. The study was carried out in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

All authors have significantly contributed to the findings reported here, and all authors have jointly conceptualized the study and agreed to the final submitted manuscript.

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REVIEW
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Combination Thyroid Hormone Replacement; Knowns and Unknowns

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Hypothyroidism is common throughout the world and readily diagnosed with thyroid function tests. Management should be straightforward but appears not to be the case. Thyroid hormone replacement with levothyroxine monotherapy is the standard treatment which is effective in the majority of cases. However, 10–15% of patients established on levothyroxine do not feel their health is entirely restored and some patients prefer the addition of liothyronine. Proponents of liothyronine argue that the ratio of T3 and T4 hormones is substantially altered on T4 monotherapy and therefore both hormones may be needed for optimal health. This remains controversial as clinical trials have not demonstrated superiority of combination therapy (levothyroxine and liothyronine) over levothyroxine monotherapy. There is now a pressing need for further studies and in particular randomized controlled trials in this area. To help design and facilitate dedicated trials and better understand thyroid hormone replacement, this review summarizes the evidence where there is established knowledge and agreement (knowns) and areas where research is lacking (unknowns). Agreements include the extent of dissatisfaction with levothyroxine monotherapy, biases in testing for hypothyroidism and prescribing levothyroxine, as well as variable thresholds for prescribing levothyroxine and challenges in liothyronine dosing. The review will also highlight and summarize the unknowns including the long-term safety profile of liothyronine, and potential biomarkers to identify individuals who might benefit most from combination therapy.

Keywords: combination, liothyronine, levothyroxine, hypothyroidism, treatment

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INTRODUCTION

Hypothyroidism is common throughout the world and particularly affects females (1). It is readily identifiable and treatable, but if untreated or poorly managed can have profound adverse effects (1, 2). Levothyroxine (LT4) is the current standard treatment although liothyronine (LT3) and desiccated thyroid extract (DTE) are also used (2, 3). Prior to the 1970's both combination thyroid hormone replacement (LT3 + LT4) and DTE were widely prescribed (4). The discovery that T4 is largely activated to T3 outside of the thyroid within target tissues (5) and concerns regarding dosing and stability of FT3 profiles in patients on LT3 or DTE have made LT4 the standard of care for nearly 50 years. Thyroid hormone replacement is now an important global public health issue and as at present, LT4 is the most commonly prescribed medication in the USA and the third most commonly prescribed in the UK (2).

The diagnosis and monitoring of hypothyroidism is largely biochemical and undertaken using easily available laboratory thyroid function tests. The inverse relationship between thyroid hormone and the pituitary derived hormone, thyrotropin (TSH) has lead to TSH becoming the accepted marker of thyroid status (6) and the goal of treatment is therefore to normalize TSH levels (2). Whilst such an approach appears at first instance to be straightforward several issues have been raised in recent years which necessitate further research. These are fundamental and relate to the diagnosis, treatment, and monitoring of thyroid function. As a result, calls have been made that the focus on TSH rather than symptom relief should be re-assessed (7). A joint consideration of TSH, patient symptoms, and a more personalized approach may therefore be required to address the recent surge in patient complaint rates (7). Indeed thyroid hormone, once thought to be the easiest hormone to replace, has become very controversial, with patient groups challenging the assumptions of endocrinologists.

The most pertinent issue is that there are widespread reports of dissatisfaction with LT4 replacement (8) estimated at 10–15% of patients (2, 9). It is difficult to assess the extent to which this reflects dissatisfaction in the general population as undergoing thyroid function testing itself is a predictor of psychological morbidity (10). This effect may be further exaggerated by the dramatic increase in thyroid function testing and initiation of LT4 at lower TSH thresholds in recent years (11, 12).

LT4 monotherapy results in individuals having relatively lower FT3 and higher FT4 levels than individuals with an intact, undisturbed, hypothalamopituitary-thyroid (HPT) axis (13), but the clinical relevance of this finding is controversial (14). Consistently many patients (∼20%) may be apparently "over-replaced" with LT4 as evidenced by a low or suppressed TSH (11, 15, 16). There is evidence that a lower TSH can result in greater patient satisfaction (17), but also greater long term risks (18). In the UK in particular, due to widespread concern and media coverage over recent large increases in costs of LT3 and access to treatment (19) more patients are now aware of and desiring combination LT3:LT4 replacement. Increased awareness and self-medication with LT3 has also been observed in Denmark (20) and the USA (8). Four systematic reviews/meta-analyses of trials of combination LT3:LT4 replacement found no clear benefit of combination therapy over LT4 monotherapy (21–24). However, some of the key limitations of these trials (discussed further below) included small sample sizes, variable quality in outcomes assessed, the use of only once daily ("pulsatile") rather than slow release T3 dosing and the acceptance that feedback to TSH is the same with all forms of thyroid hormone replacement resulting in potential for dose titration errors. Furthermore, there is recent evidence that T4 inhibits the deiodinase that converts T4 to T3 in target tissues, with the potential to result in paradoxically reduced levels of intracellular T3 when there is a high circulating T4/T3 ratio (25).

Based on this uncertainty regarding the benefit of LT3, current European Thyroid Association guidance (26) recognizes that LT4 is the treatment of choice but recommends that a 3-months trial of LT3 could be considered in patients with persistent unexplained symptoms despite good compliance. Likewise, a British Thyroid Association position statement also endorses LT4 as the treatment of choice but acknowledges that a trial of combination therapy could be warranted in patients who have unequivocally failed to respond to LT4 following a discussion with the patient of the uncertain risks and benefits of combination therapy (27). Whilst current American Thyroid Association guidance (28) indicates a trial of LT3 can be considered in carefully selected patients it also highlights no consistent superiority of combination LT3:LT4 therapy. DTE was not endorsed in either the European, British, or American guidelines (26–28).

Set against the uncertain benefits of combined LT3:LT4 therapy a balance has to be made against the potential for adverse consequences of using LT3. The lack of clarity in both these areas results in difficult decisions for patients and clinicians. Equally, maintaining the status quo is not desirable given the dissatisfaction that exists with LT4 monotherapy and the numbers of patients being over-replaced on current monitoring with TSH. A substantial number of patients seek to try LT3 monotherapy or other preparations such as DTE and have sought to source their therapy themselves (19). Failure of endocrinologists to engage sufficiently with these patients may place individuals at unnecessary risk of over-replacement.

Taken together there is a pressing need for further randomized controlled trials in this area, as well as research into longer acting LT3 preparations, better markers which predict tissue thyroid status, better assessments of treatment outcomes, and improved knowledge of the benefit-risk profile of long-term combination LT3:LT4 therapy. A review of the current evidence for combination thyroid hormone replacement with an overview of future research directions is therefore timely. We will summarize and identify areas of existing knowledge and research agreement (the knowns) as well as areas where data are more urgently needed (the unknowns).

THE KNOWNS

Serum T4, Not Serum T3 Is the Main Source of T3 Within Cells

In mammals, the majority of active thyroid hormone (T3) within cells is derived not directly from T3 in the circulation, but indirectly from T4, via the action of the D2 deiodinase (29, 30). The concentration of circulating T4 is about five times higher than T3 and it is the circulating T4 rather than T3 that serves as the main source of thyroid hormone for the body (5). In addition a proportion of serum T3 is also derived from serum T4 (5). These considerations have been used as a key theoretical argument against the use of LT3 in the management of hypothyroidism.

However, serum thyroid hormone levels may not entirely reflect intracellular thyroid hormone status. Via variation in uptake, activation and metabolism of T4, cells can modulate intracellular T3 levels independently of circulating thyroid hormone levels. Downstream control by thyroid

hormone transporters such as MCT8 and MCT10, the deiodinases (DIO2,DIO3), and transcription co-factors locally modify thyroid hormone bioavailability and action at the intracellular level. The potential for dichotomy between serum thyroid hormone levels and intracellular action is dramatically illustrated in reports of individuals with rare mutations in the MCT8 transporter (Allan-Herndon-Dudley syndrome) or the thyroid hormone receptor alpha (present in many tissues but not the hypothalamus/pituitary) (31, 32). Here, marked tissue-specific hypothyroidism is seen with dramatic effects on bone and brain development despite "normal" or even raised serum thyroid hormone levels (31, 32). The effects of more common variants are less clear.

At the same time evidence has been accumulating of tissue-specific regulation of thyroid hormone contents in tissues via differential expression of thyroid hormone transporters and iodothyronine deiodinases (29, 33). It has been hypothesized that LT4 monotherapy may not restore intracellular T3 levels in the brain in all patients and this may explain the dissatisfaction some patients have on LT4.

There Is Dissatisfaction in Individuals With Hypothyroidism Who Are on LT4 Monotherapy

Even with normalization of TSH, patients may still complain of symptoms overlapping with overt hypothyroidism such as lethargy, sleepiness, memory problems, inability to concentrate, and process information ("brain fog"), feeling cold and weight gain (2). Symptoms consistent with the presence of hypothyroidism are very non-specific and cannot be used to differentiate those with hypothyroidism from euthyroid controls (34). Dissatisfaction with LT4 monotherapy is not a new issue. Even in the early 1970's when traditionally higher treatment doses (150 mcg of LT4 and 45 mcg of LT3) were used and treatment initiated at more advanced disease stages, it was recognized that a subset of individuals required LT3 to restore health (35). This would suggest that dissatisfaction is not simply a consequence of persistent symptomatology due to minor thyroid function abnormalities. It is a consistent finding that patients on LT4 replacement even with a normal TSH display significant impairment in psychological well-being compared to controls of similar age and sex (36–39). Whilst this could, at least in part be attributed to disease misclassification (not treating co-existing depression) or confounding it could also indicate that in a proportion of patients there is imperfect replacement with LT4 alone.

For cohort studies and clinical trial design, it is worth considering that standard assessments such as the General Health Questionnaire and Hospital Anxiety and Depression Score may not capture effectively all the symptoms leading to dissatisfaction. Thus, more disease-specific outcome measure such as THYPRO (40) may provide more robust outcome assessment.

Patients Who Have Their Thyroid Function Tested Are Growing in Number, but Are Not Entirely Representative of the General Population

There has been a steady increase in thyroid function testing in the last two decades (1, 11, 12, 41). In keeping with this, cohort data from Norway has identified that the prevalence of untreated hypothyroidism is 0.1% representing an 84% fall from the 1990s (42). Individuals who have their thyroid function checked are more likely to be female, aged over 60 (43), have higher psychological morbidity (10), but do not appear to have increased rates of hypothyroidism compared to the general population (10). In the UK, the three most common reasons for thyroid function testing that led to a prescription of LT4 are depression, fatigue and weight gain (11). This suggests—perhaps not surprisingly—that individuals with these complaints are preferentially selected for testing for thyroid function and since subclinical hypothyroidism is common (especially in females over 60 where rates approach 10%), a "co-incidental" finding of subclinical hypothyroidism with these symptoms will commonly occur.

Importantly, these potential selection biases will need to be taken into account in cohort studies. Symptoms at initial levothyroxine prescription may need to be considered by selection criteria or minimization depending on the outcome measure used.

Patient Characteristics Influence Current Prescribing Practice and Treatment Course

Individuals with only borderline abnormalities in TSH are increasingly being started on LT4 (11, 12, 44). It appears that thresholds for levothyroxine initiation differ according to patient symptoms at presentation. For example, individuals with tiredness or depression had levothyroxine initiated at lower TSH thresholds than those who had their thyroid function checked as part of general screening (11). This is pertinent as it is well-established that thyroid-related patient complaints overlap with a plethora of non-specific symptoms which may be caused by other conditions (11, 45, 46). Future trials of combination thyroid hormone replacement need to have inclusion criteria which take into account the patient's thyroid status at initiation of levothyroxine. For instance, individuals with non-specific symptoms and borderline or normal thyroid function (prior to treatment) may have little to gain from either LT4 or combination LT3:LT4 therapy (47, 48).

Being female and having higher net family income are predictive factors for receiving LT4 (49) although it is not clear from this data if this was driven by increased thyroid function testing in these groups. In the UK there is a 49-fold variation in the number of LT3 prescriptions per 1,000 LT4 prescriptions by local prescribing region (19). A substantial driver of this has been the dramatic price rise in LT3 in the UK. Crucially, patients in deprived areas were much less likely to receive LT3, even before the price rises, and this has become more dramatic following prescription cost changes. Cohort studies looking at long term

outcomes of LT3 therapy will need to consider social class bias when assessing these outcomes.

Whilst data on the demographics of patients on LT3 are lacking, insights may be attained from studying cohorts of patients on LT4. Individuals treated with LT4 for primary hypothyroidism in the UK were more likely to develop a suppressed TSH following treatment if they originally presented with depression, tiredness or fatigue (11). Women and individuals with a TSH of <4.0 mU/l at index prescription were also more likely to develop a suppressed TSH (11) on LT4 while older individuals and those with cardiovascular risk factors were less likely to (11). It is not unreasonable to suggest that if these symptoms have persisted despite supra-physiological doses of LT4, then these individuals may be more likely to receive a prescription for LT3. This creates two issues, (i) a potential bias in individuals who receive LT3 and (ii) inclusion into LT3 trials of individuals with borderline thyroid function (including top of the normal range) who become classified as hypothyroid because they are receiving LT4. In such individuals LT3 may be of questionable benefit thus negating a treatment response. There is therefore a compelling argument that in the inclusion/exclusion criteria setting, an index TSH threshold before *initial* LT4 prescription should be used; a TSH threshold between 6.0 and 10.0 mU/l at index LT4 prescription may balance the competing priorities of ease of recruitment and ensuring the presence of sufficient thyroid disease.

Patients on Levothyroxine Replacement Do Not Have "Normal" Thyroid Function

Patients treated with levothyroxine to achieve normal serum TSH values typically have serum triiodothyronine (T3) concentrations in the reference range but with increased levels of T4 resulting in a significantly increased T4/T3 ratio (13, 50). In individuals studied before and after thyroidectomy absolute serum T3 levels change little once TSH is normalized (50, 51), but in individuals with subclinical hypothyroidism where there is increased deiodinase activity and T3 production, treatment with T4 alone results in a fall in absolute T3 serum levels in addition to the rise in T4/T3 levels (52).

Studies of patients established on levothyroxine also reveal that the relationship between TSH and FT3 disassociates on LT4 monotherapy particularly in athyreotic individuals (13, 50, 53). Supra-physiological levels of FT4 are actually required to normalize serum FT3 levels (50). In animals LT4 monotherapy was unable to restore euthyroidism at the tissue levels despite bringing TSH within its reference range (54) and supra-physiological doses were also required to normalize tissue T3 levels (54). In a small number of patients with goiter who received levothyroxine before thyroidectomy, a 33% increase in LT4 dose was required after thyroidectomy to maintain pre-surgical TSH levels (51) but a clear reduction in FT3 was not observed in the same patient before and after thyroidectomy.

It has been reported in cell lines that T4 inhibits the activity and promotes the destruction of the key deiodinase enzyme, D2 that activates T4 to T3 within cells (55). This results in "autoregulation" that serves to protect cells from excess T4, but also to increase local T3 production where T4 levels are reduced. If this mechanism occurs *in vivo*, treating subclinical hypothyroid subjects with T4 alone paradoxically may make their tissue hypothyroidism worse. Note that in the cell line studies, pituitary cells were less prone to such "autoregulation," suggesting that TSH would still be effectively suppressed with T4 monotherapy despite inhibition of T3 generation within peripheral tissues. As result, paradoxical reduction in local T3 generation on T4 alone would be expected to occur despite normalizing or even suppressing TSH levels. This is likely to be less of a concern in profoundly hypothyroid individuals, in whom any increase in T4 is likely to contribute positively to the generation of intracellular T3 as levels are so low, but result in "net deterioration" in individuals who were initially only subclinically hypothyroid. What is less clear is the extent of other regulators in the thyroid hormone pathway, including FT3 levels and transport. Taken together it is plausible that multiple "hits" are required to induce substantial tissue hypothyroidism, making the percentage of people who will clearly benefit from LT3 difficult to predict.

In support of this hypothesis, a recent large cohort study confirmed that individuals on LT4 had 15–20% lower T3:T4 ratios on LT4 than healthy or matched controls (14). Prolonged exposure to this different ratio appeared to have adverse health outcomes as, LT4 treated individuals had higher a body mass index despite lower calorie consumption, reported less physical activity and were more likely to be taking antidepressants (14), although confounding remains a possibility.

An additional factor is an individually tailored dose of T4 is not widely used, although more mixed doses are being used e.g., 100/125 mcg alternate days. It is also common to encounter a subset of hypothyroid patients who require unexpectedly high doses of T4 or who have erratic control. A variety of factors can cause this including poor compliance, use of different brands, impaired absorption secondary to gastro-intestinal pathology (e.g., coeliac disease), use of other medications (e.g., iron supplements) timing of medication (ingestion of caffeine around the time of T4 will impair absorption) (56, 57). Repeated dose changes and sub-optimal control will also reduce satisfaction with T4. Novel T4 preparations including gel and elixir may improve absorption (58) and may therefore improve satisfaction with LT4 treatment.

Reliance on TSH Alone May Be Problematic

Due to small changes in thyroid hormone levels resulting in large TSH modifications, TSH has been the preferred diagnostic test for hypothyroidism (6). However, this approach has been challenged (7). Key issues include that its reference-interval is not universally agreed and it is not usually adjusted for other factors such as ethnicity and iodine status. Studies have also identified in athyreotic rats treated with LT4 that the brain, skeletal muscles and liver continue to exhibit markers of hypothyroidism (25). In contrast, in the hypothalamus the effect of T4 on downregulating D2 activity appears to be less as discussed above, which might explain TSH normalization despite tissue hypothyroidism elsewhere. This is important as it

is perhaps unlikely therefore that TSH accurately reflects thyroid hormone concentrations in all tissues and organs on T4 alone. By contrast, chronic combination LT3:LT4 therapy was able to normalize thyroid hormone dependent biological parameters in the brain, liver, and skeletal muscle (25).

Restoring TSH to the reference-range has been adopted as the goal of thyroid hormone replacement. However, TSH may not be corrected to an individual's own reference range. Genetic variation in PDE8B is robustly associated with TSH levels (59–61) but this relationship is lost in individuals on LT4, thus indicating that whilst TSH is normalized on LT4 therapy this may not be to an individual's genetic set-point (60). It is well-established that intra-individual variation in thyroid hormone levels is much narrower than inter-individual variation (62) and that thyroid hormone levels in healthy adult individuals are largely genetically determined (59, 61). Thus, individuals could have thyroid function measurements within the normal laboratory reference range which are "abnormal" for them. The clinical implications of this remain unclear, but are pertinent in individuals on thyroid hormone replacement who may attain blood thyroid hormone levels within the reference-range but not at their genetically determined set-point (60). In practice, severe clinical presentations of hypothyroidism have been documented in individuals with only mildly elevated TSH levels (63). Given an individual has a narrow genetically determined set point of thyroid function (62) the long-term health consequences are unclear and merit further study.

Current LT3 Dosing Strategies Do Not Replicate T3 Levels in Euthyroid Individuals

FT3 levels show a substantial peak 2–4 h after a dose and wear off after 12 h in individuals on a single daily dose of LT3. Similar profiles on FT3 are observed in hypothyroid patients on combination LT3:LT4 therapy (64), LT3 monotherapy (65, 66), and also euthyroid individuals taking LT3 (67). These profiles are markedly different from euthyroid individuals not on thyroid medications. In particular the peak FT3 level is often substantially above the reference-range. Long-acting T3 preparations are in development and may be the key here (68). Long-acting preparations make it easier to monitor dosing, as it possible to assess 24 h T3 exposure from a single measurement. In addition, TSH levels under these state T3 (and T4) conditions are also more likely to be a true reflection of thyroid status compared to the feedback effects of "pulsatile" T3 on TSH levels (66)—see "unknowns" below. Normalization of tissue thyroid status in murine studies was achieved using slow release pellets of T4 and T3 (25). It is worth noting that in contrast to LT3 novel LT4 formulations including gel and elixir are available in most but not all countries (58).

The ratio of LT3:LT4 is also of paramount importance. Given the majority of circulating T3 comes from peripheral conversion of FT4 to FT3, a secretion ratio of FT4:FT3 in healthy individuals is ∼14:1 (69, 70). ETA guidelines (26) suggested three possible methods for calculating LT3 and LT4 doses to give a T4:T3 ratio between 13:1 and 20:1 which is close to normal thyroid hormone secretion, however this ratio is generally lower than those used in clinical trials to date (70). Using these recommended ratios (26) a patient on 100 mcg of LT4 would require between 4 and 6 mcg of LT3 which would be impractical to deliver in split doses with current LT3 formulations.

LT3 dosing is also critical when assessing potential benefits. It is worth noting that one of the key early studies which favored LT4 monotherapy in terms of patient preference over LT3:LT4 combination treatment (71) used up to 60 mcg of LT3 a day. Anxiety and palpitations were common at this high dose which may explain why LT4 monotherapy was preferred in this study.

Combination LT3:LT4 Therapy Has Not Demonstrated Clear Superiority Over LT4 Monotherapy in Clinical Trials

To date there have been at least 13 randomized controlled trials (RCT) comparing efficacy of combination LT4:LT3 therapy vs. LT4 monotherapy (70). Four systematic reviews/meta-analyses (21–24) of these trials of combination LT3:LT4 replacement found no clear benefit over LT4 monotherapy in terms of mood, health-related quality of life, or cognitive function. This data has been taken to suggest that at the population level there is no benefit of using combination therapy over monotherapy. However, in no study to date has physiological replacement been achieved (59). It is noteworthy that Wiersinga et al. (26) reviewed the five cross-over trials (72–76) and identified that despite adequate blinding 48% of all hypothyroid patients preferred the LT3:LT4 combination therapy, 27% preferred LT4 monotherapy, and 25% had no preference. Furthermore, studies in which low or low normal levels of TSH were achieved frequently reported benefit (73, 77–79). Although this has been taken to suggest "overdosing," the lower TSH levels may relate to the unphysiological nature of the T3 replacement as discussed above. Potentially more robust disease-specific patient reported outcomes such as THYPRO are now available (40). Overall seems reasonable to assume that combination therapy cannot be assumed to have no advantages over monotherapy until a well-designed long-term trial of physiological combination treatment with a sustained release preparation or three times daily dosing of FT3 has been conducted. Such a trial ideally would be adequately powered to assess specific subsets of patients e.g., those with symptomatic disease, established hypothyroidism, and genetic predispositions such as variation in DIO2 (80).

UNKNOWNS

Serum TSH May Be Determined Predominantly by Circulating T4, Not T3

While the complex inverse relationship between the thyroid hormones and TSH (6) is well-established, the relative contribution of FT4 and FT3 in regulating TSH levels is less clear. FT4 may have a greater effect on TSH than FT3 (81). As a result, feedback on serum TSH and its production may be predominantly determined by circulating T4, not T3. This becomes highly relevant when the balance between T4 and T3 in the circulation is perturbed by replacement with T4 monotherapy, as is standard in endocrine practice. Thus,

TSH levels may appear to be suppressed more easily on LT4 monotherapy. More research is needed to clarify the relative contributions of LT4 and LT3 therapy on TSH levels.

The Effect of Short Acting LT3 May Have a Disproportionate Effect on TSH

T4 is slowly metabolized with a serum half-life of several days. As a result, once daily dosing results in <20% variation in T3 levels over 24 h. In contrast, T3 has a shorter half-life, and even 2–3 times a day dosing results in substantial fluctuation in T3 levels over 24 h (64). This variation increases with the proportion of T3 in the replacement formulation when combination T3/T4 therapy is used. The effect of this is unclear, but one potential consequence is that spikes of T3 may have a greater suppressive effect on TSH than for the same area under the curve for T3.

The Role of Circulating T3 Is Unclear

Circulating T3 appears to have a different role in physiology than circulating T4. This is illustrated by several observations, some familiar to endocrinologists and some less familiar. First, even during profound hypothyroidism (e.g., T4 < 5.0 pmol/L, TSH > 20 mU/L), T3 is almost always maintained in the reference range. Second, by contrast, during acute illness T3 falls rapidly whereas T4 is maintained, or may even rise (82). Third, 25% of children under 7 years of age have T3 levels above the adult reference range, with a normal TSH (81). Fourth, higher T3 levels in children (and adults) correlate with *increased* body mass index (83). Consistent with this, children with higher T3 levels have earlier onset of puberty (81). Mendelian randomization studies suggest that in fact fat mass somehow causes an increase in T3 levels, rather than vice-versa (83). Taken together, these observations suggest that circulating T3 levels have a role in signaling nutritional or health status to the brain, perhaps more so than being a major source of T3 to tissues.

The Usefulness of SNPS in Predicting Who Will Benefit From Combination Therapy Is Unclear

Genetic variation in the deiodinases is associated with altered thyroid function and adverse health outcomes (84). However, effects have been modest and not consistently replicated. Individuals with genetic variants in *DIO2* (rs225014) (80) and MCT10 (rs12885300) (77) have shown preference for combination LT3:LT4 therapy with an additive effect (77). However, these studies had limitations due to multiple testing (80) and small sample size (77). Individual SNPs may have poor discriminatory power in determining who might benefit from LT3:LT4 combination therapy. Alternative strategies such as a panel of single nucleotide polymorphisms or the use of thyroid hormone metabolites or metabolomics may provide better insight into tissue thyroid status. Metabolomic profiles are likely to represent a unique method to assess thyroid status as the metabolites released into the serum provide a fingerprint of thyroid hormone action in tissues. Small studies have shown FT4 concentrations are strongly linked to serum acylcarnitines and phosphatidylcholines, indicating enhanced transport of fatty acids to mitochondrion and subsequent β-oxidation (85). More recently a larger study of urinary metabolites revealed positive relations of alanine, trigonelline, and lactic acid with FT4 and negative relations of dimethylamine, glucose, glycine and lactic acid with TSH (86). To date the effect of FT3 on metabolomics has not been studied. This is likely to be an important research area with potential to identify new biomarkers related to thyroid status.

There Is Limited Data About the Safety Profile of Long-Term LT3 Therapy

An important argument against the widespread use of LT3 is the lack of data on the safety of long-term use. Given the relatively small number of patients using combination LT3:LT4 therapy this is unlikely to be resolved soon. Most of the studies have been of short duration and only one lasted a full year. A recent observational study (87) of 400 patients on LT3 therapy (median duration of therapy 10.9 years) did not identify an increased risk of cardiovascular disease (HR 1.04 95%CI 0.70, 1.54), atrial fibrillation (HR 0.91 95%CI 0.47, 1.75), or fractures (HR 0.79 95%CI 0.49, 1.27), after adjusting for age, despite lower levels of TSH in "ever users" of T3 vs. "never users" (1.07 mU/l vs. 2.08 mU/l, $p < 0001$). An increased risk of anti-psychotic medication prescription was noted in T3 users, which may reflect biases in individuals who receive LT3. It is also worth noting that none of the 13 RCTs comparing combination therapy to LT4 monotherapy showed any increase in adverse events in the combination group, although follow-up was short ranging from 5 to 52 weeks (70).

It would nonetheless be prudent to have agreed monitoring protocols in individuals on long-term LT3 therapy. Sensible assessments would include pulse rate and rhythm, blood pressure, and mood (in particular anxiety). Tests to be considered will include ECG, echocardiograms, and DXA to assess bone density. Having agreed standardized monitoring protocols, ongoing regular assessments in patients on LT3 will be needed to both address and monitor for potential safety concerns.

CONCLUSION

It has proved challenging when treating a proportion of patients with hypothyroidism to ensure they are restored to optimal health. This is perhaps surprising considering the high global prevalence of this chronic condition (1). Although current management practices may satisfy the majority of patients, they remain sub-optimal. Key issues affect all stages of management and include excessive thyroid function testing, variation in threshold for treatment by presenting symptoms, widespread over-replacement on LT4 as evidenced by suppressed TSH, and inequality in access to LT3 (19). Using TSH alone to make management decisions may not be effective for at least a sub-group of patients (7). All these factors result in challenging decisions and different priorities for patients and their physicians (88, 89).

Given the importance of thyroid status for health (90) and the substantial dissatisfaction with LT4 replacement (8)

further research is urgently needed. There also needs to be studies addressing alternative markers of tissue hypothyroidism other than TSH, such as genetic markers, metabolites, and metabolomic markers. In tandem there needs to be cohort studies of existing patients on combination therapy to identify any potential long-term health complications.

Clinical trials will need to factor in the following to have the best chance of a meaningful result: (i) patient selection to take into account symptoms at first diagnosis, TSH threshold at LT4 initiation, co-morbidities including auto-immune diseases, (ii) Near physiological LT3 therapy in the trials (multi-dosing or long acting LT3) titrated to a physiological T4/T3 ratio and a normal or low normal TSH, (iii) appropriate outcome assessment such as THYPRO, (iv) sufficient power to detect modest effects which would still be of substantial impact at the population given the prevalence of hypothyroidism and dissatisfaction with LT4 therapy, and (v) sufficient power to detect treatment effects in subgroups, such as patients with the DIO2 genotype.

AUTHOR CONTRIBUTIONS

PT, CD, and OO drafted and revised the article. VE, IM, and AS revised the article.

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Predicting Optimal Combination LT4 + LT3 Therapy for Hypothyroidism Based on Residual Thyroid Function

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Objective: To gain insight into the mixed results of reported combination therapy studies conducted with levothyroxine (LT4) and liothyronine (LT3) between 1999 and 2016.

Methods: We defined trial success as improved clinical outcome measures and/or patient preference for added LT3. We hypothesized that success depends strongly on residual thyroid function (RTF) as well as the LT3 added to sufficient LT4 dosing to normalize serum T4 and TSH, all rendering T3 levels to at least middle-normal range. The THYROSIM app was used to simulate "what-if" experiments in patients and study designs corresponding to the study trials. The app graphically provided serum total (T4) and free (FT4) thyroxine, total (T3) and free (FT3) triiodothyronine, and TSH responses over time, to different simulated LT4 and combination LT4 + LT3 dosage inputs in patients with primary hypothyroidism. We compared simulation results with available study response data, computed RTF values that matched the data, classified and compared them with trial success measures, and also generated nomograms for optimizing dosages based on RTF estimates.

Results: Simulation results generated three categories of patients with different RTFs and T3 and T4 levels at trial endpoints. Four trial groups had >20%, four <10%, and five 10–20% RTF. Four trials were predicted to achieve high, seven medium, and two low T3 levels. From these attributes, we were able to correctly predict 12 of 13 trials deemed successful or not. We generated an algorithm for optimizing dosage combinations suitable for different RTF categories, with the goal of achieving mid-range normal T4, T3 and TSH levels. RTF is estimated from TSH, T4 or T3 measurements prior to any hormone therapy treatment, using three new nonlinear nomograms for computing RTFs from these measurements. Recommended once-daily starting doses are: 100 μg LT4 + 10–12.5 μg LT3; 100 μg LT4 + 7.5–10 μg LT3; and 87.5 μg LT4 + 7.5 μg LT3; for <10%, 10–20%, and >20% RTF, respectively.

Conclusion: Unmeasured and variable RTF is a complicating factor in assessing effectiveness of combination LT4 + T3 therapy. We have estimated and partially validated RTFs for most existing trial data, using THYROSIM, and provided an algorithm for estimating RTF from accessible data, and optimizing patient dosing of LT4 + LT3 combinations for future combination therapy trials.

Keywords: simulation, combination therapy, levothyroxine, liothyronine, residual thyroid function, hypothyroidism etiology

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INTRODUCTION

Combination therapy for hypothyroidism using both levothyroxine (LT4) and liothyronine (LT3) continues to be a topic of much interest to physicians and patients alike (1–4). This interest has been spurred, in part, by the well-documented finding that the ratio of total thyroxine (T4) to total triiodothyronine (T3) increases during LT4 therapy, compared with endogenous euthyroidism (5), and also that T3 levels may be lower than in the native state (6). Furthermore, animal studies suggest T3 deficiency at the tissue level with LT4 therapy alone (7, 8). This interest persists despite the generally mixed results of combination therapy trials, with most results not demonstrating a benefit of such therapy in terms of improvement in quality of life, mood, or neurocognitive function, but some patients expressing preference for therapy containing LT3 (9–22). When examining outcomes of either quality of life, mood, or neurocognitive function, trials fall into 3 broad categories: those showing substantial clinical benefit of combination therapy (11, 16), those showing partial benefit based on some outcome measures, but not others (10, 13, 18, 21), and those showing no benefit (9, 12, 14, 15, 17, 19, 20, 22). Similarly, the seven trials that examined patient preference for combination therapy can be divided into two groups: those in which patients preferred the LT3-containing therapy (9–11, 13, 16), and those in which there was no preference (18, 22).

Numerous suggestions have been offered for why these combination therapy trials did not provide evidence of clinical benefits or greater patient preference. In addition to non-physiologic thyroid hormone ratios, potential shortcomings include use of once daily LT3 therapy rather than two or three times a day therapy, or short duration trials or underpowered trials (23). Examining these trials aggregated into meta-analyses (24–26) also has not revealed benefits of combination therapy, perhaps due in part to the heterogeneity of the trial populations and methods, which include different doses of LT4 and LT3 employed, etiology of hypothyroidism, unknown degree of residual thyroid function (RTF), treatment duration, different thyrotropin (TSH), free or total T4 and T3 levels achieved in the two groups, and the outcome measures employed (23). The current work is focused primarily on degree of residual thyroid function, which we postulate may be responsible for generating quite variable responses to and perceived effects of added exogenous LT3.

The THYROSIM app (27) is a freely accessible, well-validated and mechanistically-based simulator of human thyroid hormone and TSH regulation dynamics, developed and implemented as a facile web-based and personal device application. THYROSIM has a simple and intuitive user interface for teaching and conducting simulated "what-if" experiments, graphically providing temporal dynamic responses—namely levels of serum total T4, T3, free T4 (FT4), and free T3 (FT3), as well as TSH responses over time, to various simulated system and input perturbations in 70 kg humans (28, 29). It has also been modified to predict LT4 and LT3 replacement in pediatric patients (30), used to explore TSH dynamics in primary and secondary hypothyroidism (31), and applied to LT4 bioequivalence studies (29, 32). Furthermore, the utility of the app in clinical research also has been demonstrated more recently by predicting the potentially pathophysiological effects of over-the-counter thyroid supplements (33).

In order to gain insight into the mixed results of the 14 combination therapy trials, we developed the following two hypotheses to test predictively using the THYROSIM app and retrospectively using data from the trials. For both hypotheses, combination therapy is understood to mean addition of LT3 to LT4 dosing; and "success" of combination therapy was defined as benefit in terms of improved clinical outcome measures (quality of life, mood, or neurocognitive function) or patient preference for the added LT3.

Working Hypothesis 1

Success with combination therapy will be greatest when the daily LT4 dose fraction is sufficient to normalize serum TSH and T4 and the daily LT3 dose added renders serum T3 levels within the middle to upper normal range.

Working Hypothesis 2

Success with combination therapy depends strongly on a patient's RTF as well as the LT3 added to sufficient LT4 dosing. Little or no success is predicted when RTF is 20% or more unless the daily LT3 dose added generates serum T3 levels in the mid-normal to high normal T3 range. Preference for combination therapy is not likely unless the added T3 generates high-normal range to supra-physiologic T3 levels.

METHODS

Dosage Response Simulations

The THYROSIM app (27) has been applied in the current work by exploring THYROSIM responses to exogenous LT4 and combination LT4 + LT3 hormone dosage inputs in simulated patients with primary hypothyroidism, and patients with different degrees of RTF, rendered hypothyroid by autoimmune thyroid disease, radioactive iodine therapy, external beam radiotherapy, or thyroid surgery. In support of predicted results, simulation conditions—namely dosages and predicted RTF—were adjusted to and compared with data from several studies of patients receiving synthetic combination LT4 + LT3 therapy in comparison with LT4 therapy alone (9–22). An example of a simulation matching data from Siegmund et al. (20) is shown in **Figure 1**.

RTF Measures

To obtain RTF estimates for our data with the THYROSIM app, we simulated patient dosing input regimens and output responses with serum TSH, T4, and T3 presumed to be measured before any therapy was begun. RTF is estimated by manually adjusting the T4 and T3 secretion rates on the graphic interface of the THYROSIM app, by trial-and-error. The goal is to find the best RTF (% secretion rates) that generates starting values (initial hormone concentrations) that approximate both the initial T4, T3, and TSH concentrations measured prior to dosing therapy (combination therapy or T4 monotherapy), and the approximate final concentrations measured at the end of the study period.

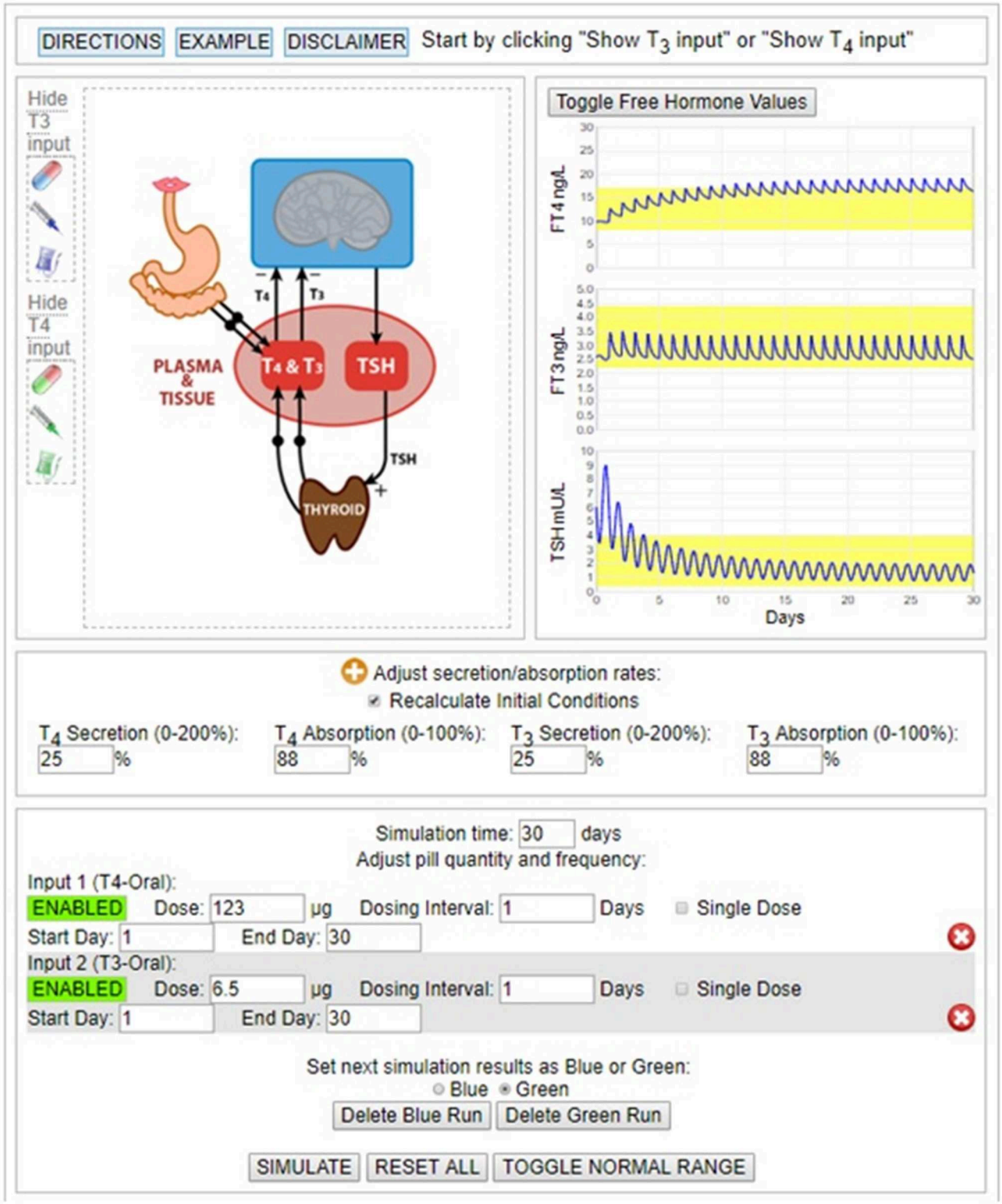

FIGURE 1 | Simulation of 123 µg LT4 + 6.5 µg LT3 dosing experiment in Siegmund 2004 trial (20). Final hormone values achieved with ~25% RTF.

For hypothyroid patients with different etiologies of their hypothyroidism, it is important that these thyroid variables are assessed after they reach a steady state, after they plateau, and after the degree of RTF also stabilizes. For example, following complete thyroidectomy, the thyroid hormone and TSH levels 6 weeks later should indicate 0% RTF. For someone with Hashimoto's hypothyroidism, in order to predict their likely non-zero RTF, at least 6 weeks are needed for the thyroid hormone and TSH levels to stabilize following likely incomplete thyroid destruction.

Only one study was available from among the 14 combination therapy trials that provided any measured patient hormone values prior to initiating therapy for hypothyroidism, and this was only for TSH (16). THYROSIM simulations were conducted with different RTF values, by varying the thyroidal T4 and T3 secretion rates from 0 (athyreotic) to 50% and recording the starting values for total serum TSH, T4 and T3. We assumed that T3 and T4 secretion rates (adjustable on the THYROSIM interface) are suppressed or reduced together by relatively the same amounts.

RESULTS

Method Validation

To help validate our computational modeling approach, we simulated the combination therapy dosing and dose-response conditions reported in the study of 10 patients from the Saravanan trial, which provided 24 h hormone profiles of TSH, FT3 and FT4 in 20 hypothyroid patients taking either LT4 monotherapy or combined LT3/LT4 therapy (34). Simulation response results (solid blue lines) are shown graphically in **Figure 2**, superimposed over the published data corresponding to these results. They match the data quite well. In particular, the ∼40% rise in mean FT3 values, peaking at ∼4 h, is well represented by the simulation and is shown to remain within the normal FT3 range (yellow band), thus tracking the previously reported data well. In comparison, the Saravanan sub-study (34) reported 3 of 10 patients in the LT3/LT4 group, but none in the LT4 alone group, had FT3 levels above their laboratory reference range at some time over the 24-h period, but lasting only for <2 h.

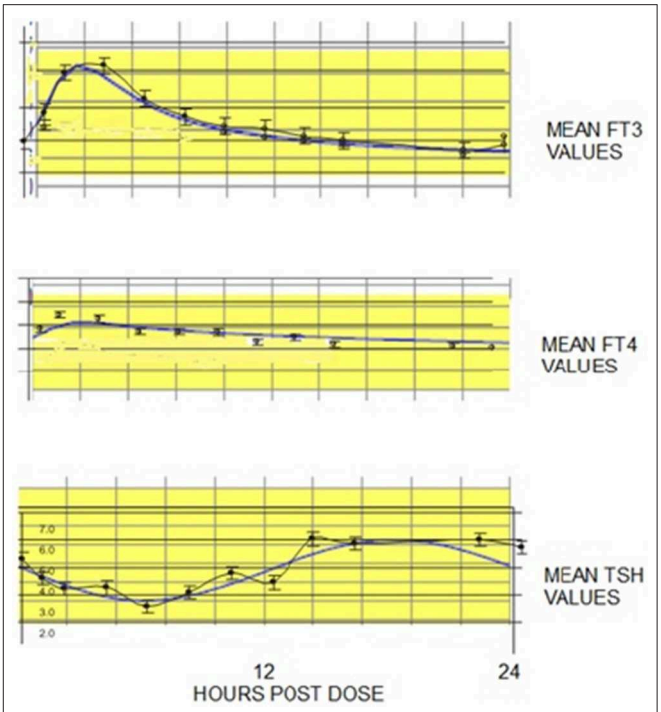

FIGURE 2 | FT3, FT4, and TSH 24 h temporal responses (blue curves) predicted by THYROSIM for simulated 117 μg T4 + 10 μg T3 dosing, superimposed over corresponding LT3, LT4, and TSH data (black dots with error bars) collected over 24 h, in 10 patients from a larger study in hypothyroid patients on combined LT3/LT4 therapy (18–34). Yellow bands represent normal ranges.

Addressing the Hypotheses

A summary of conditions, patient populations and hypothyroidism etiologies from 13 combination therapy trials (excluding Valizadeh) is given in **Table 1**. This table shows (where available) the LT4 doses prior to randomization, and the LT4 and LT4/LT3 doses in the monotherapy and combination therapy arms. Full information about the etiology of the hypothyroidism was not provided in all trials.

Predicted TSH, T4, and T3 Levels vs. RTF Values at Diagnosis

The three graphs shown in **Figure 3** illustrate the predicted TSH, T4, and T3 levels prior to initiating any therapy for hypothyroidism in individuals with RTF varying between 0% (athyreotic) up to 50% RTF. The relationships are nonlinear, particularly in the most likely RTF range, up to 30%; and TSH—followed by T4–followed by T3 levels, are the most sensitive to increasing RTF.

RTF Estimates and Predicted Success of LT3/LT3 Therapy

Patient RTF Values

The best results predicted by THYROSIM and supported by the trial data suggest that, because the trials included patients with different etiologies of hypothyroidism, the participants had varying degrees of RTF. **Table 2** shows the various trials separated into categories of simulated high, medium and low RTF values, respectively. The data in published results were incomplete, so the categories may not be completely accurate.

For the first category of high RTF (>20%), no benefit of combination therapy was predicted with respect to quality of life, mood or neurocognitive benefit or LT4/LT3 preference in the 4 trials with high RTFs (9, 10, 13, 20). We predicted that with >20% RTF, small amounts of added LT3 would have less of an impact, as assessed by various outcome measures or patient preference. We speculated that combination therapy would only be clinically successful in the setting of high RTF if the LT4 dose maintained T4 and TSH in their normal ranges and the added LT3 dose generated higher than normal T3 levels. For higher LT3 doses,

TABLE 1 | Summary of 13 trials of synthetic combination with LT4/LT3 therapy compared to LT4 alone.

References	Treatment dosing	Dose of LT4 pre-trial	Dose of LT4 in LT4 gp (number of patients)	Dose of LT4 in LT3 gp (number of patients)	Dose of LT3 in LT3 gp	Etiology primary hypo-thyroidism (number of patients)	Design	Number of patients randomized (completed follow-up)	Treatment duration	Baseline & end of study TSH differences between groups
Appelhof et al. (9)	T4: usual dose LT4/LT3: 10:1 or 5:1 ratio of T4 to T3 ratio, respectively Dosing: Twice daily for both LT4 & LT3	1.46 μg/kg/day (placebo) 1.61 μg/kg/day (LT4: LT3 10:1) 1.73 μg/kg/day (LT4: LT3 5:1)	100 μg (50 μg given twice daily)	75 μg (10:1 ratio) 75 μg (5:1 ratio) (approx. 37.5 μg given twice daily)	7.5 μg (3.75 μg twice daily) (10:1) 15 μg 7.5 μg twice daily (5:1)	Autoimmune (other causes excluded), 80% positive TPO antibodies	Parallel, blinded	141 (130)	15 weeks	Baseline TSH values 1–1.1. LT4 vs. LT4/LT3 (10:1) vs. LT4/LT3 (5:1) 0.64 vs. 0.35 vs. 0.07 (TSH lower in the 5:1 T3:T4 dose group)
Bunevicius et al. (11)	T4: usual LT4/LT3: usual T4 dose minus 50 μg/day with T3 12.5 μg/day Dosing: Once daily	175 μg (all) 181 μg (placebo first) 169 μg (LT3 first)	175 μg	125 μg	12.5 μg	Mixed—Autoimmune (16), thyroid cancer (17)	Cross-over, blinded	35 (33)	5 weeks	Baseline TSH 0.3–1.5. LT4 0.8 vs. LT4/LT3 0.5. NS‡ difference
Bunevicius et al. (10)	T4: usual LT4/LT3: usual T4 dose minus 50 μg/day with T3 10 μg/day Dosing: Once daily	All: 100 μg (7) 150 μg (3)	115 μg (approx.)	65 μg (approx.)	10 μg	All Graves disease, history of subtotal thyroidectomy	Cross-over, blinded	13 (10)	5 weeks	Baseline TSH 1.02. LT4 0.45 vs. LT4/LT3 0.47. NS‡ difference
Clyde et al. (12)	T4: usual LT4/LT3: usual T4 dose minus 50 μg/day with T3 15 μg/d Dosing: Twice daily LT3, LT4 once daily	131 μg (placebo) 136 μg (LT3) 1.6 μg/kg/day (placebo) 1.8 μg/kg/day (LT3)	131 μg (including 25 μg BID, balance given once daily)	86 μg once daily	15 μg (7.5 μg twice daily)	Mixed – Autoimmune (31), post-RAI* (10), thyroid surgery (1), post-EBRT**(1), thyroid cancer (1)	Parallel, blinded	46 (44)	4 months	Baseline TSH 2.2–2.6. LT4 2.1 vs. LT4/LT3 2.0. NS‡ difference
Escobar-Morreale et al. (13)	T4: 100 μg/day LT4/LT3: LT4 75 μg/day and T3 5 μg/d Dosing: Once daily	100 μg (all)	100 μg	75 μg 87.5 μg (add on)	5 μg 7.5 μg (add on)	Mixed – Autoimmune (23), post-RAI* (5)	Cross-over, blinded	28 (26)	8 weeks	Baseline TSH "normal". LT4 1.95 vs. LT4/LT3 2.56. LT4/LT3 > LT4

(Continued)

TABLE 1 | Continued

References	Treatment dosing	Dose of LT4 pre-trial	Dose of LT4 in LT4 gp (number of patients)	Dose of LT4 in LT3 gp (number of patients)	Dose of LT3 in LT3 gp	Etiology primary hypo-thyroidism (number of patients)	Design	Number of patients randomized (completed follow-up)	Treatment duration	Baseline & end of study TSH differences between groups
Fadeyev et al. (14)	T4: 1.6 μg/kg/day LT4/LT3: estimated T4 dose minus 25 μg/day with T3 12.5 μg/day Dosing: Once daily†	50–125 μg (?)	100 μg (25) 125 μg (7) 75 μg (9) 50 μg (1)	75 μg (median) 75 μg (10) 100 μg (4) 50 μg (2)	12.5 μg	All autoimmune	Parallel, unblinded	58 (58?)	6 months	Baseline TSH "normal". LT4 1.35 vs. LT4/LT3 1.7. NS‡ difference
Kaminski et al. (15)	T4: 125 or 150 μg LT4/LT3 75 μg + 15 μg T3 Once daily	125 or 150 μg	125 or 150 μg	75 μg	15 μg	Mixed – Autoimmune (23), post-RAI* (3), thyroid cancer (6)	Cross-over, blinded	32	8 weeks	Baseline TSH 0.31. LT4 0.19 vs. LT4/LT3 0.64 NS‡ difference
Nygaard et al. (16)	T4: usual LT4/LT3: usual T4 dose minus 50 μg/day with T3 20 or 50 μg/day, respectively Dosing: Once daily	129 μg (all)	131 μg	77 μg	20 μg	Autoimmune (all positive TPO antibodies)	Cross-over, blinded	68 (59)	12 weeks	Median TSH at diagnosis 43–82, baseline TSH 1.1, LT4 0.99 vs. LT4/LT3 0.76. NS‡ difference
Rodriguez et al. (17)	T4: usual LT4/LT3: usual T4 dose minus 50 μg/day with T3 10 μg/day Dosing: Once daily†	121 μg (all) 118 μg (seq1, placebo) 121 μg (seq2, LT3)	118 μg	121–50 μg = 71 μg	10 μg	Mixed—Autoimmune (23), post-RAI* (4), thyroid surgery (3)	Cross-over, blinded	30 (27)	6 weeks	Baseline TSH 1.7-1.8. LT4 2.5–2.9 vs. LT4/LT3 3.3–7.6. NS‡ difference

(Continued)

TABLE 1 | Continued

References	Treatment dosing	Dose of LT4 pre-trial	Dose of LT4 in LT4 gp (number of patients)	Dose of LT4 in LT3 gp (number of patients)	Dose of LT3 in LT3 gp	Etiology primary hypothyroidism (number of patients)	Design	Number of patients randomized (completed follow-up)	Treatment duration	Baseline & end of study TSH differences between groups
Saravanan et al. (18)	T4: usual LT4/LT3: usual T4 dose minus 50 μg/day with T3 10 μg/day Dosing: Once daily	123 μg (placebo) 127 μg (LT3)	123 μg	127–50 μg = 77 μg	10 μg	Primary hypothyroidism (72%?, 44% TPO antibodies), no thyroid cancer	Parallel, blinded	697 (573)	12 Months (outcomes assessed 3 and 12 months)	Baseline TSH 0.84-0.85. LT4 0.79 vs. LT4/LT3 1.25 at 12 months. LT4/LT3 > LT4 at 3 months
Sawka et al. (19)	T4: usual LT4/LT3: 50% usual T4 dose with T3 total 25 μg/day (12.5 μg BID) Dosing: Twice daily T3, once daily T4	120 μg (placebo) 132 μg (LT3)	118 μg	67 μg	19 μg (9.5 μg twice daily)	Primary hypothyroidism, excluded: thyroid cancer, history of hyperthyroidism, thyroidectomy	Parallel, blinded	40 (33)	15 weeks	Baseline TSH 1.75-2.2. LT4 1.7 vs. LT4/LT3 1.8. NS‡ difference
Siegmund et al. (20)	T4: usual LT4/LT3: usual T4 dose minus 5% with T3 5% (aim 14:1 ratio LT4 to T3) Dosing: Once daily†	100 μg (5) 125 μg (12) 150 μg (8) 175 μg (1)	129 μg	123 μg	6.5 μg	Mixed – Autoimmune (2), post-RAI* or thyroid surgery (24)	Cross-over, blinded	26 (23)	12 weeks	Baseline TSH 1.72. LT4 1.5 vs LT4/LT3 0.5. LT4/LT3< LT4
Walsh et al. (22)	T4: usual LT4/LT3: usual T4 dose minus 50 μg/day with T3 10 μg/day Dosing: Once daily†	136 μg	136 μg	86 μg	10 μg	Mixed—Autoimmune (94), post-RAI* (4), thyroid surgery (12), no thyroid cancer	Cross-over, blinded	110 (101)	10 weeks	Baseline TSH 1.3-1.5. LT4 1.5 vs. LT4/LT3 3.1. LT4/LT3 > LT4

† *Dosing not reported, assume once daily.*

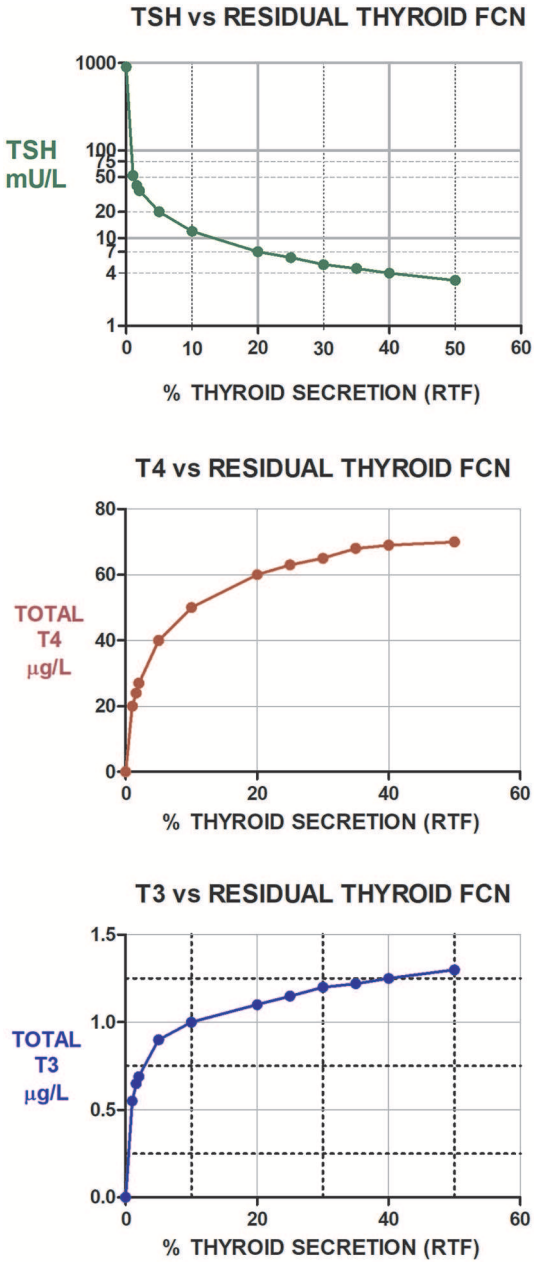

FIGURE 3 | Illustration of the predicted (and non-linear) TSH, T4, and T3 levels prior to initiating any therapy for hypothyroidism in individuals with RTFs varying between 0% (athyreotic) up to 50% RTF (hemi-thyroidectomized). Notably, as RTF increases from about 1–10%, TSH levels fall by ~80%, while T4 levels increase by ~163% and T3 levels increase by ~79%. Similarly, T4 increases by ~30%, T3 by 20% and TSH falls about 58% as RTF increases from 10 to 30%. TSH levels shown in green, T4 levels shown in red, and T3 levels shown in blue.

the effect became noticeable and combination therapy was more likely to be preferred, albeit potentially toxic.

For the second category of medium RTF (10–20%), some benefit with respect to quality of life or mood or neurocognitive benefit was predicted in the five relevant trials (12, 14, 15, 17, 19). However, the impact of the modest amount of added LT3 on outcome measures was expected to be minimized by the endogenous RTF. If RTF was low (<10%), combination therapy was predicted to provide substantial quality of life or mood or neurocognitive benefit and/or to be preferred by patients in the 4 relevant trials (11, 16, 18, 22).

Successful Therapy Based on Improved Outcome Measures

Table 3 shows the same 13 trials separated into three categories: (a) those showing substantial improvement in outcomes with combination therapy (11, 16); (b) those showing partial benefit based on some outcome measures, but not others (13, 18); and (c) those showing no benefit (9, 12, 14, 15, 17, 19, 20, 22). These same trials are also shown in **Figures 4A–C**, which categorize the trials by RTF and show the associated trial results displayed as Venn diagrams.

Successful Therapy Based on Treatment Preference

Treatment preference was assessed in 7 of the 13 trials. **Table 4** lists these trials in two categories: (a) trials in which a preference for combination therapy was expressed by participating patients (9–11, 13, 16); and (b) trials in which there was no patient preference for combination therapy (18, 22). These trials are also indicated in **Figures 4A–C**, showing how preference is related to degree of RTF, and whether either improved outcomes, preference, or both improved outcomes and preference were demonstrated in the same trial. Additionally, indicated on the figure is whether the T3 levels were predicted to be low, medium or high during the trial.

Testing Hypothesis 1

For our first hypothesis that achievement of medium-high T3 levels along with sufficient LT4 in the dose is needed for successful (improved outcomes or preference) combination therapy, our prediction was mostly correct. The Appelhof, Bunevicius, and Nygaard studies (9–11, 16) were predicted to have high T3 levels and were "successful" (see **Figures 4A,C**). The Escobar-Morreale, Kaminski, Clyde, Sawka, Rodriguez, Saravanan, and Fadeyev trials (12–15, 17–19) (**Figures 4A–C**) were predicted to have medium T3 levels and therefore the RTF might also impact their success. The Walsh and Siegmund trials (20, 22) (**Figures 4A,C**) were predicted to have low or low medium T3 levels and did not report improved outcomes or preference.

Testing Hypothesis 2

With respect to our second hypothesis of the degree of RTF (while also taking the T3 levels achieved into account) affecting the success of combination therapy, results of this prediction are shown in **Figure 4**. **Figure 4A** shows the studies with high RTF and three out of four studies are correctly predicted as not showing combination therapy to be successful. **Figure 4B** shows the studies with medium RTF and all five studies are correctly predicted as not showing combination therapy to be successful. For the prediction that low RTF would be associated with successful combination therapy due to the more noticeable

TABLE 2 | Measured mean TSH, FT4, T4, T3, FT3 values at beginning and end of trials for monotherapy vs. combination therapy groups in trials grouped according to whether patients were estimated to have high RTF (>20%), medium RTF (10–20%), or low RTF (<10%).

Trial	TSH before	TSH end	FT4 (ng/dl) before	FT4 end	T3 (ng/dl) before	T3 end	FT3 (pg/dl) before	FT3 end	T3 & *T4* ranges by sim	LT3 dose (μg)	LT4 dose (μg)
High RTF (>20%)											
Appelhof T4	1.0	0.64	1.15	1.18	111	111	–	–	–		
Appelhof T4 + T3 (10:1)	1.1	0.35	1.15	1.02	109	119	–	–	Med *Med*	7.5	75
Appelhof T4 + T3 (5:1)	1.0	0.07	1.18	1.00	115	143	–	–	Med-High *Med*	12.5	75
Bunevicius, 2002, T4	1.02	0.45	1.61	1.64	–	227	–	–	–		
Bunevicius, 2002, T4 + T3	1.02	0.47	1.61	0.96	–	247	–	–	High *High*	10	65
Escobar-Morreale T4	nl	1.95	–	1.61	–	–	–	332	–		
Escobar-Morreale T4 + T3 (5 μg)	nl	2.56	–	1.31	–	–	–	325	Med *Med*	5	75
Escobar-Morreale T4 + T3 (7.5 μg)	nl	1.09	–	1.34	–	–	–	384	Med *Med*	7.5	87.5
Siegmund T4	1.72	1.5	1.72	1.62	–	–	332	294	-		
Siegmund T4 + T3	1.72	0.5	1.72	1.56	–	–	332	324	Low- Med *High*	6.5	123
Medium RTF (10–20%)											
Clyde T4	2.2	2.1	1.2	1.2	96	87	–	–	–		
Clyde T4 + T3	2.6	2.0	1.3	0.8	89	135	–	–	Med *Med*	15	86
Fadeyev T4	–	1.35	–	1.45	–	–	–	273			
Fadeyev T4 + T3	–	1.7	–	0.96	–	–	–	267	Med *Med*	12.5	75
Kaminski T4	0.31	0.19	1.26	1.64	93	103	–	–	–		
Kaminski T4 + T3	0.31	0.64	1.26	1.03	93	98	–	–	Med -High *Med*	15	75
Sawka T4	2.2	1.7	1.30	1.38	–	–	280	286	–		
Sawka T4 + T3	1.75	1.8	1.22	0.82	–	–	267	306	Med *Low*	19	67
Rodriguez T4	1.7–1.8	2.5–2.9	*11–11.2*	*10.8–10.9*	76–79	73–86	–	–	–		
Rodriguez T4 + T3	1.7–1.8	3.3–7.6	*11–11.2*	*7.6–8.2*	76–79	95–104	–	–	Med *Med*	10	71
Low RTF (<10%)											
Bunevicius, 1999, T4	0.3	0.8	2.0	2.3/*15.2*	–	87	–	–	–		
Bunevicius, 1999, T4 + T3	1.	0.5	1.9	1.8/*11.3*	–	117	–	–	High *Med-High*	12.5	125
Nygaard* T4	1.1	0.99	–	–	–	–	–	–	–		
Nygaard* T4+T3	1.1	0.76	–	–	–	–	–	–	High *Low*	20	77
Saravanan T4	0.87	0.79	1.62	1.57	–	–	248	234	-		
Saravanan T4 + T3	0.85	1.25	1.64	1.14	–	–	248	239	Med *Med-High*	10	77
Walsh T4	1.4	1.5	1.19	1.21	–	–	221	241	-		
Walsh T4 + T3	1.4	3.1	1.19	0.89	–	–	221	228	Low-Med *Med*	10	86

*Gray shading indicates T4/T3 arm of study, * study reports only free T4 index and FT3 index and does not report either total or free T4 or T3, blue font is total T4 levels in mcg/dL. "T3 and T4 levels by sim" are the levels predicted by simulation for the combination therapy group, rather than measured T3 and T4 levels, and are categorized into 3 groups (high/medium/low). Mean/median LT3 and LT4 doses in the combination therapy group are also shown.*

effect of the added LT3, we showed in **Figure 4C** that four out of four studies were correctly predicted in this category.

Recommendations for Combination Therapy Dosing in Patients Previously Untreated With T4 or T3

Our estimates of RTF allow us to make predictions regarding the dosing of LT3 that should be optimal when designing a combination therapy trial. Serum levels of TSH, T4 and/or T3 should be obtained at the time of diagnosis, either from patient history data or anew, prior to initiating any therapy and one or more (preferably >1) of the graphs in **Figure 3** can then be used as nomograms to estimate RTF. We would then predict that the following practical daily dosing combinations would serve best for starting dosing in 70 kg individuals with computed RTFs in the three given ranges. (These recommended dosages should be adjusted for body weight or other anthropomorphic measures.) To maximize compliance, once-a-day dosing responses are simulated in **Figures 5A–C** for <10% RTF, 10–20% RTF, and

TABLE 3 | Thirteen trials of monotherapy vs. combination therapy, categorized according to whether patients experienced benefits or not during combination therapy.

Benefit as assessed by improved outcomes
a) Substantial quality of life or mood or neurocognitive benefit
Bunevicius, 1999
Nygaard
b) Some quality of life or mood or neurocognitive benefit
Escobar-Morreale (5 μg T3)
Escobar-Morreale (7.5 μg T3)
Saravanan*
c) No quality of life or mood or neurocognitive benefit
Appelhof (T4 + T3, 10:1 ratio)
Appelhof (T4 + T3, 5:1 ratio)
Bunevicius, 2002
Clyde
Fadeyev
Kaminski
Rodriguez
Sawka
Siegmund
Walsh

*Showed benefit at 6 months but not at 12 months.

TABLE 4 | Seven trials of monotherapy vs. combination therapy, categorized according to whether patients preferred combination therapy or not.

Therapeutic preference
a) Preference for combination therapy
Appelhof (T4 + T3, 10:1 ratio)
Appelhof (T4 + T3, 5:1 ratio)
Bunevicius, 1999
Bunevicius, 2002
Escobar-Morreale (5 μg T3)
Escobar-Morreale (7.5 μg T3)
Nygaard
b) No preference for combination therapy
Saravanan
Walsh

>20% RTF. This should keep T3 excursions within the normal range, as shown in the figures. If individual patient clinical requirements warrant, the LT4 + LT3 dosages can be split in half and prescribed 2x a day, with smaller excursions in serum T3, as shown in **Figure 5D**.

$$\begin{aligned}
\text{For RTFs} < 10\% &\rightarrow 100\ \mu\text{g LT4} + 10-12.5\ \mu\text{g LT3, once daily}\\
\text{For RTFs } 10-20\% &\rightarrow 100\ \mu\text{g LT4} + 7.5-10\ \mu\text{g LT3, once daily}\\
\text{For RTFs} > 20\% &\rightarrow \text{LT4} = 87.5\ \text{ug} + 7.5\ \text{ug LT3, once daily}\\
\text{For RTFs} > 20\% &\rightarrow \text{LT4} = 50\%\ \text{of } 87.5\ \text{ug}\\
&\quad + \ 50\%\ \text{of } 7.5\ \text{ug LT3, twice daily.}
\end{aligned}$$

DISCUSSION

Our two working hypotheses are reasonably well-supported by our simulation data and comparative analysis with the data from the 13 combination trials. Evidently, these hypotheses are strongly intertwined, in a complex way, probably as a consequence of the tight, nonlinear couplings and homeostatic feedback effects among these well-regulated hormones. Importantly, the T3 (and T4) levels that can be achieved during combination therapy, and whether they are low, medium or high, appear to be affected endogenously by RTF—in a nonlinear way—as well as by the exogenous LT4 and LT3 dosages given. The latter are immediately under the influence of the same endogenous regulatory system components following absorption of the dosages. Overall, if the RTF is low, the added T3 seems to provide more impact in terms of either improved outcomes or patient preference. If the RTF is high, the same dose of T3 appears to have less impact However, if the amount of T3 added is relatively high, thus achieving a high or supraphysiologic T3 level, then there also is a positive impact in terms of either improved outcomes or patient preference—with due consideration to the clinical effects of T3 toxicity.

We recognized in existing trial data that, in the presence of sufficient T4, the T3 levels needed to ensure patient preference were higher than those needed to provide improved outcome measures; and this was borne out by our analyses. This motivated our coupled hypotheses and their analysis by "what-if" simulations of the trial data. We found a similar number of studies (five studies) associated with patient preference for combination therapy (9–11, 13, 16) as those demonstrating improved outcomes (four studies) (11, 13, 16, 18). Four of the five studies that showed patient preference had high simulated (and measured) T3 levels (9–11, 16), the exception being the Escobar-Morreale study (13) in which the simulated T3 levels were mid-range.

Our analysis was limited by several complicating factors present or absent in the trial data. T4/T3 ratios reported in the various studies were very different, some with initially higher T4/T3 ratios at baseline and the T4/T3 ratios substantially lower in the combination therapy arm (15, 17). In addition, not all studies provided full laboratory values at baseline, during the study, or at the end of the study [e.g., Nygaard (16)]. In a few studies dosing regimens were not clear. One study (18) showed improved outcomes at 6 months, but not at 12 months. We classified this study as having a positive outcome, in part because all other studies were 6 months or less in duration. However, this may not be the best way to categorize this trial, which may have demonstrated a placebo effect at 6 months. The 2002 Bunevicius study (6) was not amenable to comparative analysis as the RTF appeared to be >100%; this might be because this hypothyroid trial population consisted of Graves' disease patients who had undergone surgery for their disease, which may have been incomplete, with enough residual thyroid tissue to make dosing formulation more difficult. The Valizadeh study (21) could not be simulated for unclear reasons.

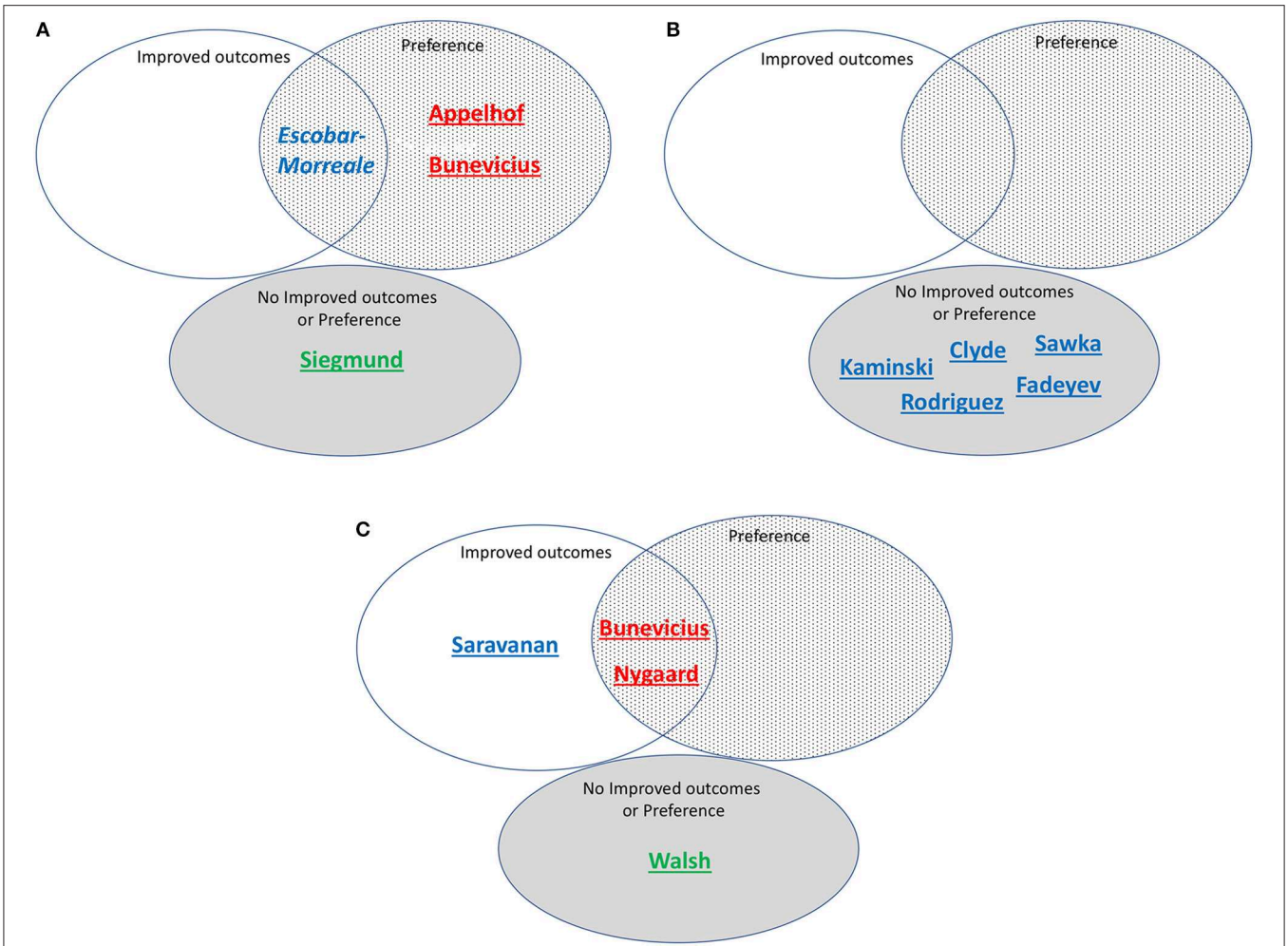

FIGURE 4 | (A) Studies assessed as including patients with high residual thyroid function (>20%) and/or T3 levels predicted by simulation, grouped according to whether improved outcomes, preference, both improved outcomes and preference, or neither improved outcomes nor preference was reported. Red font indicates high simulated T3 levels. Blue font indicates medium simulated T3 levels. Green font indicates low simulated T3 levels. Underlined indicates correctly predicted. Italics indicate incorrect prediction. Bunevicius is 2002 study. **(B)** Studies assessed as including patients with medium residual thyroid function (10-20%) and/or T3 levels predicted by simulation, grouped according to whether improved outcomes, preference, both improved outcomes and preference, or neither improved outcomes nor preference was reported. Red font indicates high simulated T3 levels. Blue font indicates medium simulated T3 levels. Underlined indicates correctly predicted. **(C)** Studies assessed as including patients with low residual thyroid function (<10%) and/or T3 levels predicted by simulation, grouped according to whether improved outcomes, preference, both improved outcomes and preference, or neither improved outcomes nor preference was reported. Red font indicates high simulated T3 levels. Blue font indicates medium simulated T3 levels. Green font indicates low simulated T3 levels. Underlined indicates correctly predicted. Bunevicius is 1999 study.

Additional limitations of prior studies that might have affected the rigor of our analyses include the following. The studies clearly included patients with different etiologies of their hypothyroidism and a wide spectrum of RTF values. There is inter-assay variability across the various studies conducted in various countries, especially for FT3 assays, making it difficult to obtain very close comparative results in all cases. Not all studies reported the timing of phlebotomy, and whether blood samples were drawn at random times of day or were trough levels, making it possible that thyroid hormone levels, particularly T3 or FT3 could vary by as much as 40% (34).

There are also limitations of the trials that, in turn, may have led to limitations in our analysis of them. With regard to patient satisfaction and patient preference issues assessed in the various trials, it must be acknowledged that many symptoms of hypothyroidism are non-specific and overlap with symptoms of other conditions (35). Therefore, it is possible that lack of improvement in quality of life, mood, or neurocognitive function noted could have occurred because reported deficits were not thyroid-related. It is also possible that improvements in mood or preference for combination therapy were reported because a different condition such as depression was in fact being treated (36, 37). In addition, some of these trials

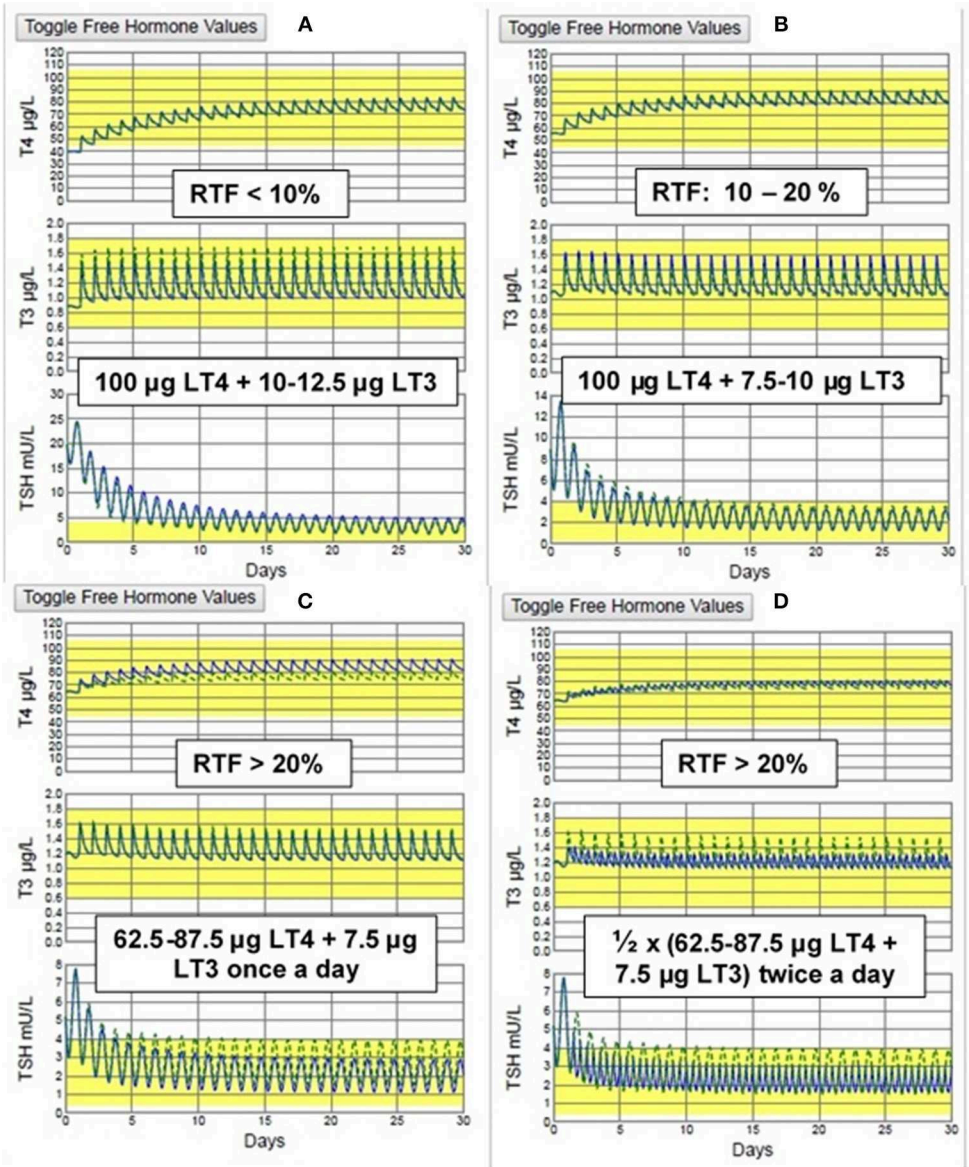

FIGURE 5 | THYROSIM simulated T4, T3, & TSH responses to the recommended dosage combinations: for **(A)** low RTF (<10%) & **(B)** medium RTF (10–20%) (TOP) & **(C)** high (>20%) RTF using once daily dosing and **(D)** high (>20%) RTF using twice a day dosing. (BOTTOM). The smallest T3 (and TSH) excursions are seen with twice daily dosing, but no values are outside the normal ranges with once a day dosing.

may have been too short to allow sufficient adaptation for either benefits to be seen or adverse effects to occur. Despite these limitations, we believe we have achieved the goal of our studies.

In summary, our results reliably support the notion that RTF differences are a key factor in explaining the ambiguities in the spectrum of combination therapy study results reported between 1999 and 2016. As added value, we have adapted our RTF estimation methodology for combined LT4 + LT3 dosing that is practical and potentially optimal when designing a combination therapy trial. Serum TSH, T4 and/or T3 levels at the time of diagnosis should be obtained from patient history data or anew, and prior to initiating any therapy; and one or more of the three graphs in **Figure 3** can then be used as nomograms to estimate RTF from individual patient data. Using this algorithm, we have provided combination dosing schemes that should serve best for starting dosing in 70 kg individuals with computed RTFs in the three given ranges. These are readily scaled by individual patient requirements, body weights or other anthropomorphic measurements.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

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

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Conflict of Interest: JD is the creator and developer of the THYROSIM app.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Treatment of Hypothyroid Patients With L-Thyroxine (L-T4) Plus Triiodothyronine Sulfate (T3S). A Phase II, Open-Label, Single Center, Parallel Groups Study on Therapeutic Efficacy and Tolerability

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Sodium salt of levothyroxine (L-T4) is the treatment of choice of hypothyroidism. Yet, L-T4 monotherapy produces supoptimal 3,5,3′-triiodothyronine (T3)/T4 ratio in serum, as compared to normal subjects, and a minority of hypothyroid individuals on L-T4 complain for an incomplete well-being. Orally administered 3,5,3′-triiodothyronine sulfate (T3S) can be converted to T3 in humans, resulting in steady-state serum T3 concentrations for up to 48 h. In this study (EudraCT number 2010-018663-42), 36 thyroidectomized hypothyroid patients receiving 100 (group A), 125 (group B), or 150 μg (group C) L-T4 were enrolled in a 75 days study in which 25 μg L-T4 were replaced by 40 μg of T3S. A significant, progressive reduction in mean FT4 values was observed, being the largest in the group A and the smallest in group C, while no relevant variations in FT3 and total T3 serum values were observed in the three groups. TSH serum levels increased in all groups, the highest value being observed in group A. Lipid parameters did not show clinically significant changes in all groups. No T3S-related changes in the safety laboratory tests were recorded. No adverse event was judged as related to experimental treatment, and no patient discontinued the treatment. Twelve patients judged the L-T4+T3S treatment better than L-T4 alone, while no patient reported a preference for L-T4 over the combined treatment.

In conclusion, the results of this study indicate that a combination of L-T4+T3S in hypothyroid subjects may allow mainteinance of normal levels of serum T3, with restoration of a physiological FT4/FT3 ratio and no appearance of adverse events. Further studies are required to verify whether the LT4+T3S chronic combined treatment of hypothyroidism is able to produce additional benefits over L-T4 monotherapy.

Keywords: hypothyroidism, L-Thyroxine, substitutive therapy, thyroid hormone metabolism, 3,5,3′-triiodothyronine sulfate

INTRODUCTION

Hypothyroidism is one of the most common endocrine disorders (1), developing as a consequence of impaired action of thyroid hormones on target tissues (2). In most instances hypothyroidism is due to insufficient secretion of thyroid hormone, turning into reduced serum concentrations of thyroxine (T4) and triiodothyronine (T3). T4 is the main product of thyroid secretion, but needs to be converted to T3 in order to bind to thyroid hormone receptors. Only a lesser fraction of circulating T3 is produced directly by the thyroid whereas the majority derives from outer-ring deiodination of T4 in peripheral tissues (3). Beside deiodination, both T4 and T3 can be metabolized by alternate pathways, mainly sulfation and glucuronidation (4, 5).

Appropriate hormonal substitutive treatment is essential to reduce morbility and mortality of hypothyroid subjects (6). Even though T3 is the active molecule, administration of sodium salt of levothyroxine (L-T4) is the substitutive treatment of choice in hypothyroidism (7), since daily oral ingestion of L-T4 warrants stable serum T3 concentrations and prevents the non-physiologic surges of T3, which occur after L-triiodothyronine (L-T3) oral intake. Nevertheless, L-T4 monotherapy produces a low T3/T4 ratio in serum, as compared to normal subjects, since the amount of T3 directly secreted from the thyroid is lacking (8–11). Furthermore, a minority of hypothyroid individuals on L-T4 complain for an incomplete well-being and about symptoms that may be reconducted to a hypothyroid status, despite having their serum TSH within the normal range. Several studies have been conducted to explore the possibility of restoring physiological concentrations of thyroid hormones by administration of a combination of L-T4 and L-T3 in various proportion, but no clear advantages could be demonstrated over the standard L-T4 monotherapy (12, 13).

We have recently demonstrated that 3,5,3′-triiodothyronine sulfate (T3S) can be converted to T3 following oral administration in humans. T3S to T3 conversion was dose dependent and, after a single dose, resulted in steady-state serum T3 concentrations for up to 48 h, suggesting that T3S might represent a new agent to be combined with L-T4 for the treatment of hypothyroidism (14). The aim of this proof of principle study was to investigate the efficacy and safety of combined administration of L-T4+T3S in hypothyroid subjects for a prolonged period.

MATERIALS AND METHODS

The study was performed at a single center (Endocrinology Unit, University Hospital of Pisa, Italy). Project management, monitoring and reporting of the study were carried out by the Contract Research Organization (CRO) ISPharm srl, Via Dorati 117, Lucca, Italy. Data management and statistical analysis were carried out by the Studio Associato Airoldi, Cicogna e Ghirri, Via Manzoni 43, Milan, Italy. Laboratory tests were performed at the Pisa Hospital, except T3 sulfate assay, which was performed at CRB/Biology, Bracco Research Center, Via Ribes 5, Colleretto Giacosa (TO), Italy.

Description of T3S synthesis, composition of pharmaceutical form and hormonal assays have been previously reported (14).

The study was performed according to a phase II, open-label, uncontrolled, single center, parallel groups study design. Inclusion criteria were: written informed consent; outpatient of either sex, aged between 18 and 70 years; total thyroidectomy for any reason, on stable (at least 3 months) substitutive or TSH suppressive L-T4 therapy (daily dose: 100/125/150 μg); no evidence of endogenous hormonal production (thyroglobulin <5 ng/mL and undetectable thyroglobulin antibody); FT_4, FT_3, Total T3 (TT3) values within the normal ranges for euthyroid subjects reliability in terms of medication compliance and capability of understanding the study protocol procedures and timelines.

Exclusion criteria were: history or current evidence of cardiovascular diseases, e.g., congestive heart failure NYHA class >1, coronary artery disease, myocardial infarction, severe hypertension, cardiac arrhythmias; history, or current evidence of significant liver (i.e., AST/ALT higher than twice the upper limit of normal range) or renal (i.e., creatinine >2 mg/dl) failure, metabolic, or endocrine diseases (e.g., uncontrolled diabetes mellitus), or any other underlying medical condition that might interfere with the study; treatment with drugs affecting thyroid hormone absorption or metabolism; any medical condition or other circumstances which would significantly decrease the chances of obtaining reliable data, achieving study objectives, or completing the study and/or post dose follow-up examinations; immunocompromised patients; malignant diseases or any other disease with life expectancy <2 years; history of alcohol abuse, drug abuse, psychological, or other psychiatric diseases that could invalidate informed consent or limit the subject compliance with protocol requirements; artificial or parenteral feeding; allergy, sensitivity, or intolerance to study drugs and/or any of study drug formulation ingredients; pregnant or breastfeeding females, or females not practicing adequate contraceptive measures; patients unlikely to comply with the protocol or unable to understand the nature, scope and possible consequences of the study; patients who received any investigational drug within the last 3 months; employees of the study center (i.e., principal investigator, sub-investigator, study coordinators, other study staff, employees, or contractors of each), with direct involvement in the proposed study, as well as family members of the employees or the investigator.

Thirty-six Caucasian thyroidectomized outpatients were enrolled and assigned to one of 3 treatment groups (12 patients for each group) according to the ongoing L-T4 dose: 100, 125, or 150 μg daily, and named group A, B, and C, respectively. Patients were consecutively enrolled until each group achieved the required number. There were no blinding procedures. The baseline characteristics of enrolled patients are reported in **Table 1**.

On average, T4 and T3 are secreted by the thyroid in a molar ratio of about 15:1, corresponding to 100 μg T4 and 6 μg T3. The amount of T3 that is directly produced by the thyroid is about 20% of daily T3 production (30 μg), meaning that 24 μg T3 are produced by peripheral deiodination of T4. Based on these assumption, 25 μg of the L-T4 dose, representing the amount of

TABLE 1 | Patients' characteristics at study entry.

		Group A (12 patients)	Group B (12 patients)	Group C (12 patients)	Overall (36 patients)
Age (years)	Min–max	32.5–68.4	32.4–65.1	26.1–57.7	26.1–68.4
	Median	50.1	56.3	47.7	50.1
	Mean ± SD	50.8± 11.4	53.1 ± 9.2	46.1 ± 8.7	50.0 ± 10.0
Gender, *N* (%)	Female	11 (92)	5 (42)	9 (75)	25 (69)
	Male	1 (8)	7 (58)	3 (25)	11 (31)
Weight (Kg)	Min–max	554–95	68–110	58–102	54–110
	Median	66	78	84	75
	Mean ± SD	68 ± 13	81 ± 13	84 ± 13	78 ± 14
Height (cm)	Min–max	154–175	160–180	160–180	154–180
	Median	165	169	171	168
	Mean ± SD	165 ± 6	170 ± 6	170 ± 6	168 ± 7
BMI (Kg/m^2)	Min–max	19.8–33.8	23.5–38.3	21.3–38.9	19.8–38.9
	Median	23.9	26.9	29.1	26.8
	Mean ± SD	25.2 ± 4.4	28.1 ± 4.3	29.0 ± 4.7	27.4 ± 4.7
Reason for thyroidectomy, *N* (%)	Thyroid cancer	9 (75)	10 (92)	9 (75)	29 (81)
	Nodular goiter	3 (25)	1 (8)	3 (25)	7 (19)
Time since thyroidectomy (years)	Min–max	0.6–15.4	1.5–31	0.4–11.3	0.4–31
	Median	3.0	5.9	4.4	5.0

T4 that by peripheral deiodination should provide 6 μg T3, was substituted with T3S. The dose of 40 μg T3S was selected on the basis of the previous study (13), as the lower dosage able to attain safe and putatively effective serum levels of FT3.

The L-T4 dose was therefore partially replaced by T3S, as follows:

Group A From 100 μg L-T4
To 75 μg L-T4 +40 μg T3S
Group B From 125 μg L-T4
To 100 μg L-T4 + 40 μg T3S
Group C From 150 μg L-T4
To 125 μg L-T4 + 40 μg T3S

The investigational product was administered together with L-T4, in the morning, after at least 12 h fasting; food intake was restrained for 20 min post-dose. During the study the L-T4 dose remained unchanged, whereas the T3S dose was suitable for decrease or increase by steps of 20 μg daily (up to 100 μg maximum daily dose) based on to the hormonal status (FT_3, FT_4, TSH), the clinical findings and the investigator opinion.

The study flow-chart is shown in **Figure 1**. After starting T3S, patients were visited every 15 days (for a maximum of 45 days) until the euthyroid state was achieved (titration period). The following control visits were performed monthly for 2 months. Routine hematology included measurement of: red blood cell count, white total and differential blood cell count, hemoglobin, hematocrit, and platelets count. Routine blood chemistry included measurement of: liver enzymes, creatinine, blood urea nitrogen, fasting plasma glucose, albumin, total protein, and electrolytes (sodium, chloride, and potassium).

The primary objective of the study was to investigate the feasibility of maintaining the euthyroid state -based on TT3, FT3, FT4, and TSH circulating levels in hypothyroid patients, by partially substituting the actual dose of L-T4 with T3S. The secondary objectives were: to assess the safety of T3S prolonged administration; to identify the optimal L-T4/T3S substitutive ratio; to compare the judgment of the patients on L-T4 vs. L-T4+T3S therapy; to evaluate the effects of the combined therapy on serum lipids profile.

The study protocol, patient information leaflet and informed consent document were approved by the reference Independent Ethics Committee (IEC) of the investigational study site (CEAVNO Comitato Etico Area Vasta Nord Ovest), all patients gave written informed consent in accordance with the Declaration of Helsinki. The study was registered at EudraCT with the study number 2010-018663-42.

STATISTICAL ANALYSIS

Statistical analysis was performed using SAS® system, PC release 9.2 (SAS Institute, Cary, US). This was a pilot study and a formal sample size calculation was not feasible. In the previous absorption study (13) a sample size of four patients for each dose regimen was able to show the gastrointestinal absorption of the T3S and the extent of this; therefore a sample size of 12 patients per dose group was judged sufficient to provide preliminary information about the therapeutic efficacy of T3S and the optimal L-T4/T3S dose ratio.

Primary efficacy variables were TT3, FT3, FT4, TSH. Secondary efficacy variables were T3S, judgment of the patients on L-T4 vs. L-T4+T3S therapy, lipid parameters. Safety variables of the study included vital signs, laboratory parameters (hematology and chemistry) and adverse events. T3S values below the detection limit, 3 ng/dL, were imputed as the limit value $3/\sqrt{2}$ after stating that the values above the limit were roughly log-normally distributed. Missing data

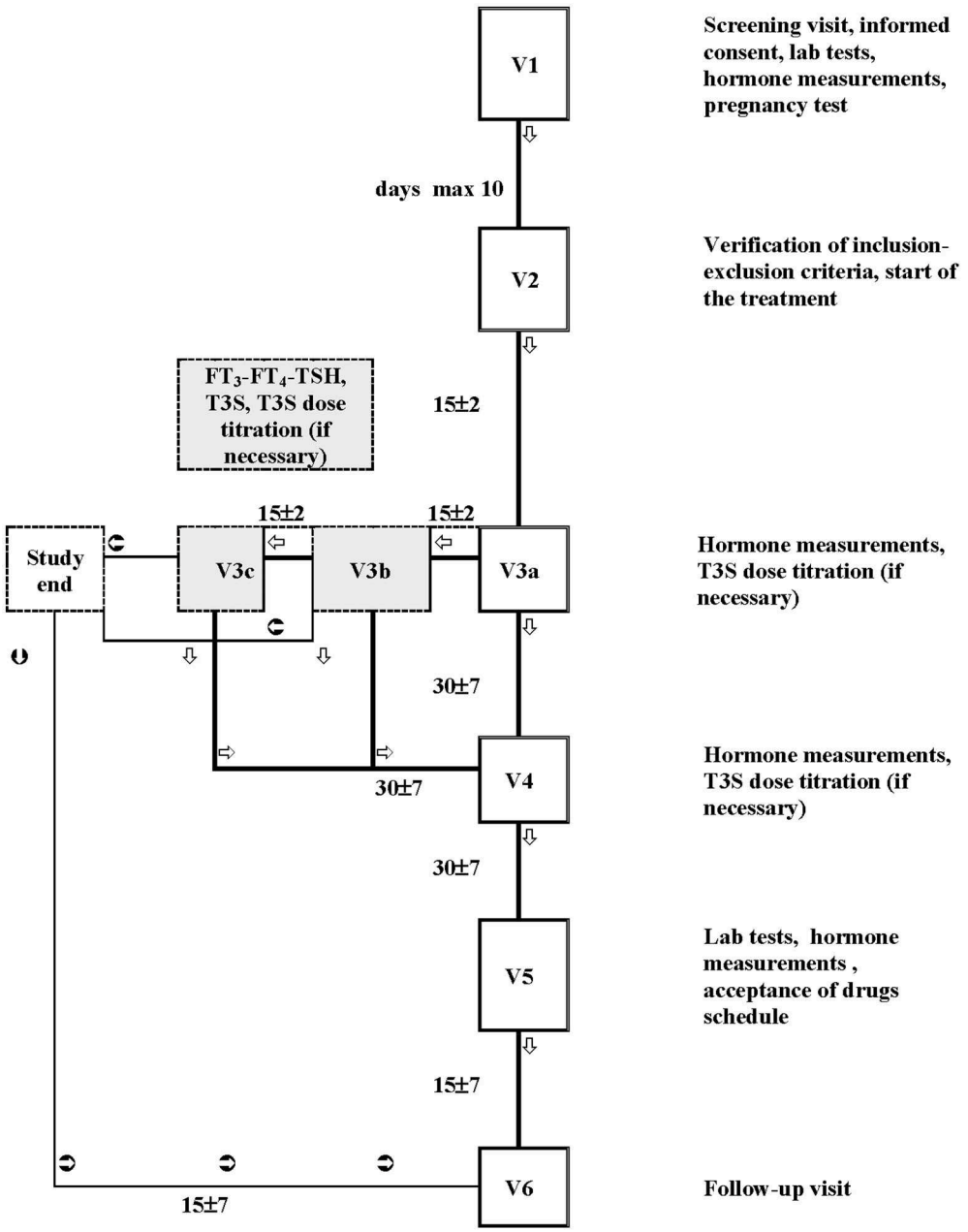

FIGURE 1 | Flow chart of the study. The study plan included a screening visit (Visit 1), in which patients potentially eligible were checked for inclusion and exclusion criteria; Visit 2 was performed within 10 days from Visit 1 to confirm the compliance with the inclusion and the exclusion criteria; if confirmed, the L-T4 therapy schedule was changed to L-T4+T3S. The next visits (max 3 visits: V3a, V3b, and V3c) were performed every 15 days and were dedicated to T3S titration. If during the titration period the patients maintained (or attained) the metabolic control (i.e., hormonal parameters in the accepted range), the T4+T3S dosage remained unchanged until the next follow-up visit (Visit 4, 1 month after), when a T3S dosage change was allowed. A fifth visit (visit 5) was performed after a further month of therapy. If at the end of the titration period (visit 3c) patients did not attained the metabolic control, they were removed from the study. Intermediate visit(s) were arranged in case of adverse events or whenever judged suitable for patient safety by the investigator. A safety follow-up visit (visit 6) was arranged 15 (+ maximum 7) days after visit 5.

were not imputed. TSH levels were markedly right-skewed and were therefore log-transformed to obtain approximately normal distributions as required to use parametric statistics. Changes from baseline of laboratory variables and vital signs were analyzed as differences, if the variable distribution was approximately normal and ratios if the variable distribution was approximately log-normal. Conventionally, $p < 0.05$ was considered statistically significant. Means of differences from V1 for T3S, TT_3, FT_3, and FT_4 levels and geometric means of ratios vs. V1 TSH levels (log-normally-distributed) were calculated. For each hormone was first analyzed the change from V1 to V3a, when the effect of the initial T3S dosage was measured

TABLE 2 | Serum levels of T3S, TT3, FT3, FT4 (mean and *SD*), and TSH (geometric mean and min-max values) in various groups throughout the study.

	Visits	**V1**	**V3a**	**V4**	**V5**
	Study times (days)	**0**	**15**	**45**	**75**
	n	**36**	**36**	**34**	**34**
T3S ng/dL	Group A	8.68 (3.55)	9.00 (3.55)	8.51 (5.53)	8.98 (4.48)
	Group B	10.55 (4.82)	9.33 (3.34)	13.63 (7.55)	8.55 (3.16)
	Group C	10.89 (5.65)	7.59 (5.22)	6.81 (4.76)	10.04 (7.28)
TT3 ng/dL	Group A	116 (20.1)	122 (11.4)	110 (12.8)	113 (11.5)
	Group B	108 (16.2)	123 (49.9)	111 (17.2)	105 (17.8)
	Group C	116 (15.1)	108 (14.4)	112 (20.4)	112 (21.3)
FT3 pg/mL	Group A	3.61 (0.63)	3.61 (0.61)	3.23 (0.31)	3.32 (0.57)
	Group B	3.32 (0.50)	3.57 (0.48)	3.50 (0.95)	3.42 (0.48)
	Group C	3.55 (0.40)	3.64 (0.53)	3.45 (0.32)	3.37 (0.28)
FT4 pg/mL	Group A	12.05 (1.75)	9.84 (1.33)	8.97 (1.37)	9.03 (1.52)
	Group B	11.35 (1.45)	9.72 (1.02)	9.21 (1.41)	8.84 (1.14)
	Group C	12.81 (2.05)	11.58 (1.30)	10.93 (1.37)	10.83 (1.34)
TSH μUI/mL	Group A	0.382 (0.035–3.520)	0.337 (0.670–2.170)	0.734 (0.157–7.130)	1.954 (0.580–12.700)
	Group B	0.362 (0.027–2.770)	0.365 (0.085–1.550)	0.875 (0.1167–3.370)	1.526 (0.268–7.750)
	Group C	0.402 (0.018–1.160)	0.500 (0.139–1.060)	1.040 (0.244–2.500)	1.050 (0.121–2.310)

in all 36 patients and there was no bias in comparing L-T4+T3S doses due to T3S dose adjustment in poor responders to the initial dosage. Changes (differences or ratios) from V1 to V3a were tested using Student's paired *t* test both overall and within dose groups, and their relationship with T4 dose was examined by one-way ANOVA with T4 dose as an interval variable. The subsequent analysis from V3a to V5 was restricted to the 34 patients who did not require T3S dose adjustment. Mixed-model linear regression analysis of changes (differences or ratios) vs. V1 was used, with visit and dose as fixed effects and subjects as random effects. Visit was always modeled as an interval variable, a one-unit difference representing a 1-month interval between scheduled time of visits. The covariance structure was chosen according to the Akaike criterion corrected for small samples (AICC) as compound symmetry for FT_3 and FT_4 and heterogeneous compound symmetry for T3S, TT_3, and TSH.

RESULTS

The entire population of 36 patients completed the study. Compliance was assessed on the basis of the tablets consumption, according to the prescribed dose and the treatment duration. All patients followed the scheduled drug regimen, with minimal differences between expected and actual number of returned tablets in only three patients.

Thirty-four patients maintained the same T3S dose from V3a to V5. The T3S dose was increased in 2 patients on the basis of hormonal results: in one patientfrom group A, the T3S dose was increased to 60 μg from V3a to V5; in one patient from group B, the T3S dose was increased to 60 μg from V3a to V3b, and to 80 μg from V3b to V5. The treatment time-frame at stable T3S dose was (mean ± SD) 73.6 ± 6.4 days (min 61–max 87).

Table 2 and **Figure 2** show FT4, FT3, TT3, TSH, and T3S values measured before and at various time points during T3S administration. A significant, progressive reduction in mean FT4 values was observed, being the largest in the group A and the smallest in group C ($p < 0.001$ in the pooled data), while no relevant variations in FT3 and TT3 values were observed in the three groups. As expected from FT4 reduction, TSH serum levels increased in all groups, the highest value being observed in group A ($p < 0.001$ in the pooled data). T3S levels, measured 24 h after T3S oral administration, remained unchanged throughout the entire study period.

Figure 3 shows the FT4/FT3 ratio plotted vs the L-T4/T3S dose ratio before and during L-T4+T3S treatment. The 2 patients who had to increase the T3S dose to 60 and 80 μg, respectively, are indicated as receiving a 1.25 L-T4/T3S ratio while the other groups are indicated as receiving 1.88 (group A), 2.5 (group B), or 3.13 (group C) L-T4/T3S ratio. At the start of the study the two patients with the L-T4/T3S dose ratio 1.25 were inside the reference range of the circulating FT_4/FT_3 ratio; 4/11 patients of the 1.88 group, 5/11 in the 2.50 group, and 7/12 in the 3.13 group were over the upper limit of the reference range (meaning that 45.4% of the entire population were above the normal range). At the last visit, the FT4/FT3 ratio was within the normal range in all but 1 patient in the 2.5 group and 3 patients in the 3.13 group. Therefore, after combined therapy, the FT4/FT3 ratio was within the normal range in 32/36 patients (88,9%). At V5 serum TSH was above the normal range in 2 patients from group A and one patient from group B (**Figure 4**).

No correlation was found between the T3S or the L-T4 dose and TT3 or FT_3 serum levels at the end of the study.

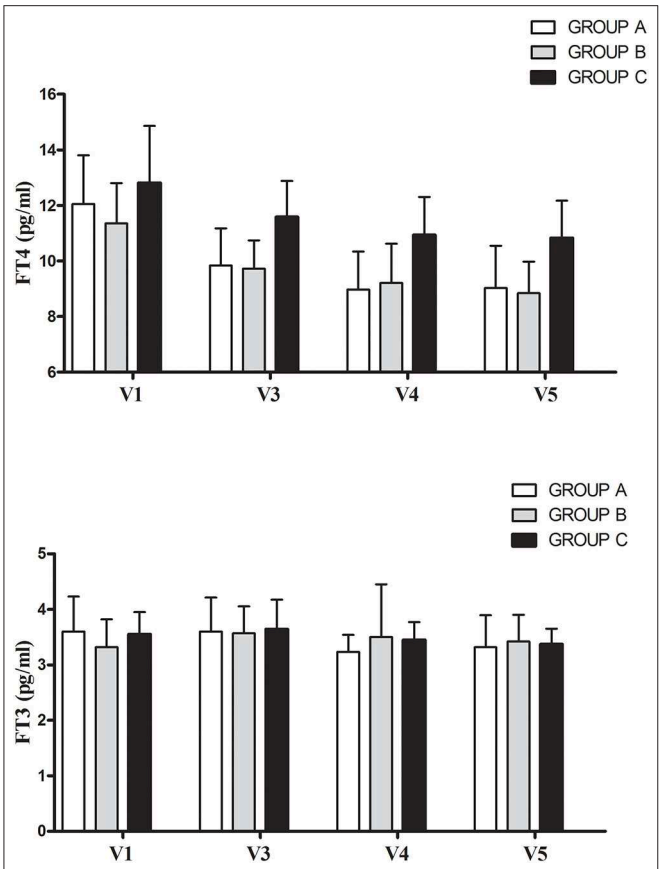

FIGURE 2 | Mean (+ *SD*) changes of free thyroid hormone before (V1) and during L-T4 + T3S therapy in the 34 patients who maintained the same T3S dose throughout the entire study. The various groups are labeled according to the T4/T3S ratio of their treatment regimen: group A = 1.88, group B = 2.5, group C = 3.13.

Lipid parameters did not show clinically significant changes in all groups.

No T3S-FT_4-FT_3-related changes in the safety laboratory tests were recorded. Four patients complained of one adverse event: upper airways inflammation, lumbar pain, chest pain and shoulder fracture due to road accident. No adverse event was judged as related to experimental treatment, and no patient discontinued the treatment.

Both in group A and group B, 5 patients judged the L-T4+T3S treatment better than L-T4 alone while 7 patients did not report differences between the treatments; in group C, the respective numbers were 2 and 10. No patients reported a preference for L-T4 vs. the combined treatment. The reason for preference of L-T4+T3S treatment included physical well-being (12 patients), psychological well-being (9 patients) and tolerability (three patients).

DISCUSSION

Sulfation of thyroid hormones is catalyzed by sulfotransferases, a family of enzymes located in the cytoplasmic fraction of several tissues, in particular liver, kidney, intestine and brain (5, 15–17). Sulfation of iodothyronines accelerates their deiodination by type I deiodinase (D1) and facilitates their excretion in the bile and urine (18). At the same time, sulfation of T3 protects the active hormone against degradation by the type 3 deiodinase (19). Therefore, while serum levels of T3S are usually very low in the euthyroid adult, they are increased in conditions where type I activity is reduced, such as the fetal life or non-thyroidal illness (20–22). T3S is biologically inactive, but enzymes capable of desulfation of T3 have been described in tissues and in the intestinal miocrobiota, and a potential role of T3S as a reservoir of T3 has been hypothesized under conditions of low D1 activity (23–26). The recent observation that pharmacological administration of T3S can produce steady concentrations of serum T3 suggested that T3S might represent a T3 derivative to be used in combination with T4 in the therapy of hypothyroidism (14).

The results of this pilot study indicate that in hypothyroid patients it is possible to partially substitute L-T4 with T3S, with no reduction of FT3 levels. As a consequence, a normal FT4/FT3 ratio was restored in most patients. The relative amount of administered T4:T3S that better reproduced a physiological hormonal profile was around 3:1. Serum TSH increased, depending on the relative amount of L-T4 that was subtracted from the dose regimen, though remained within the normal range in most patients. This observation suggests that there are no risks of developing thyrotoxicosis after administration of T3S. This consideration is supported by the peculiar metabolism of T3S that is mainly converted to inactive 3,3′-diiodothyronine sulfate by D1. D1 activity is directly regulated by T3 (27). Thus, high T3 levels accelerate T3S deiodination, leaving less T3S available for desulfation. This counter regulatory mechanism would protect against thyroid hormone excess, while promoting the activation of thyroid hormone (desulfation) if the latter is deficient. By the same mechanism, the potential influence of factors affecting D1 activity (e.g., selenium intake) might modulate T3S availability and compensate for changes in T4 to T3 conversion. As expected from the previous study (14), serum T3S, measured 24 h after oral administration, did not show relevant changes due to the rapid clearance of the sulphated iodothyronine. Since we do not have measures of thyroid hormone action in various tissues, we cannot exclude that some biological effects could be exerted in selected tissues capable of local desulfation of the T3S.

One strength of this study is that all patients were thyroidectomized with no residual functioning thyroid tissue to avoid potential interference by endogenous hormonal production. Oral T3S administration did not produce adverse side effects at any of the doses administered, and patients' acceptance appeared favorable, one third judging the L-T4+T3S treatment better than L-T4 alone and the others finding no differences. These results are of difficult interpretation and may be biased because of the unblinded design of the study. Yet, they are reassuring as for tolerability of T3S during a chronic treatment.

Additional limitations of this study include the small number of patients and lack of objective parameters to evaluate

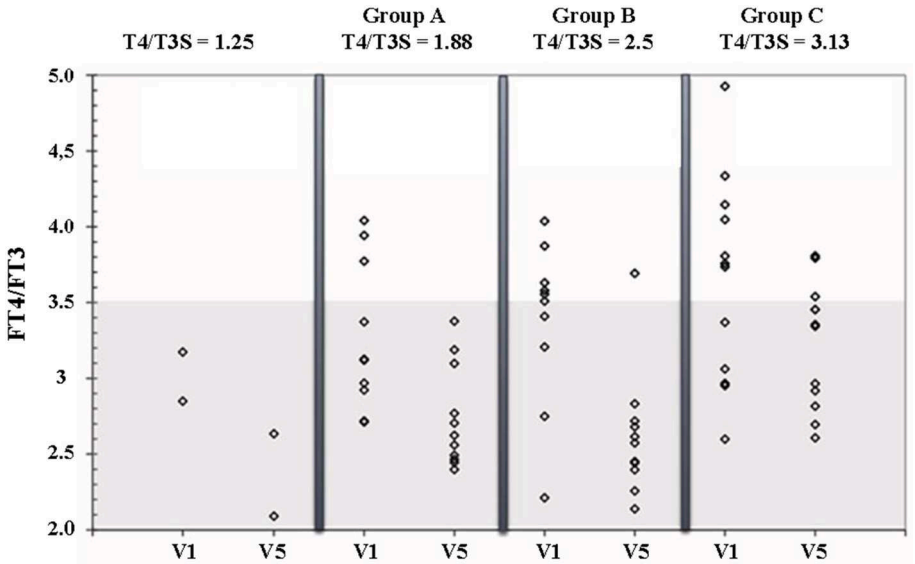

FIGURE 3 | FT4/FT3 ratio in patients on L-T4 treatment (V1) and on L-T4+T3S (V5). The various groups are labeled according to the T4/T3S ratio of their treatment regimen: group A = 1.88, group B = 2.5, group C = 3.13. The 2 patients from group A and group B, who had to increase the T3S dose to 60 and 80 μg, respectively, are indicated as receiving a T4/T3S ratio = 1.25. The shaded area represents the 95% reference range for the FT4/FT3 ratio in the normal euthyroid population.

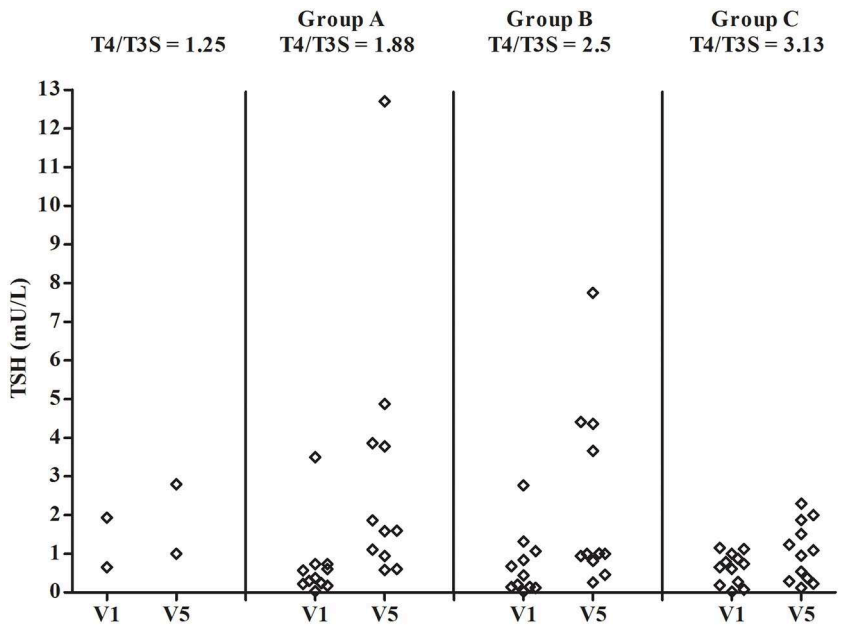

FIGURE 4 | Individual concentrations of serum TSH in patients on L-T4 treatment (V1) and on L-T4+T3S (V5). The various groups are labeled according to the T4/T3S ratio of their treatment regimen: group A = 1.88, group B = 2.5, group C = 3.13. The 2 patients from group A and group B, who had to increase the T3S dose to 60 and 80 μg, respectively, are indicated as receiving a T4/T3S ratio = 1.25.

thyroid hormone action. Yet, we believe there is sufficient evidence to promote larger studies aimed at evaluating the potential advantages of the combined T4+T3S treatment over T4 monotherapy.

In conclusion, the results of this study indicate that partial substitution of L-T4 with a combination of L-T4+T3S in hypothyroid subjects may allow maintenance of normal levels of serum T3, with restoration of a physiological FT4/FT3 ratio and no appearance of adverse events. Further studies are required to verify whether the chronic LT4+T3S combined treatment of hypothyroidism is able to produce additional benefits over L-T4 monotherapy.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by CEAVNO Comitato Etico Area Vasta Nord Ovest. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

FS conceived the study design, contributed to data interpretation, and wrote the manuscript. CP, MG, GQ, and IR carried out the patients selection and data collection. GS was involved in the study design and data managing. GC and EG were involved in the data interpretation and writing of the paper. PV contributed to the data interpretation and writing of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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frontiers in Endocrinology

OPINION
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Can Reverse T3 Assay Be Employed to Guide T4 vs. T4/T3 Therapy in Hypothyroidism?

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Keywords: reverse T3, thyroid hormones, deiodinases, T4/T3 combination, hypothyroidism, combination therapy, deiodinase 3

Among the controversial issues surrounding combination T4/T3 therapy for hypothyroidism is the choice of the best biochemical parameter by which to monitor therapy. This article explores the potential use of reverse T3 (rT3) for this role. Unsubstantiated claims in layman websites have suggested that measurement of rT3 could serve as a useful guide for monitoring combined T4 and T3 replacement therapy. A brief review of aspects of the generation of rT3 from the peripheral metabolism of T4 may place into context the possible utility of rT3 for this role.

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THYROID PHYSIOLOGY—DEIODINASES

In humans, a normal thyroid gland produces ∼85 mcg of T4 and 6.5 mcg of T3 daily (1). Thus, the ratio of T4:T3 that is directly secreted from the thyroid gland is around 13:1. The remaining daily T3 production, about 26.5 mcg, derives from peripheral monodeiodination or conversion from T4, catalyzed by the activating deiodinases type (D1) or type 2 (D2) (2). D1 catalyzes the deiodination of both the outer and inner ring of T4, while D2 is an obligate outer ring deiodinase (3). Reverse T3 is also generated from deiodination of T4 with production of ∼28 mcg/daily, but through deiodination of its inner ring by deiodinase type 3 (D3) (**Figure 1**). Because rT3 has been considered biologically inactive, deiodinase D3 represents the main physiological inactivator of thyroid hormones. It also mediates the inactivation of T3 into T2. Theoretically, the inactivation of T4 with generation of rT3 or T2 has a homeostatic role by protecting tissues from excess of thyroid hormones. Deiodinases are selenoproteins, accounting for the presence of the rare amino acid selenocysteine (Sec) in their active site (3). Adequate levels of selenium are important to the activity of all deiodinases.

Deiodinases have variable expressions among various tissues (**Table 1**). In humans, D1 is expressed mainly in the liver, kidneys, thyroid, and pituitary, while D2 is expressed in the central nervous system (CNS), pituitary, brown adipose tissue, thyroid, placenta, skeletal muscle, and heart. D1 is notably absent in the CNS. D3 is present in the skin and in the CNS, and is highly expressed in hemangiomas, fetal liver, placenta and fetal tissues (3). The high expression of D3 in the human placenta and fetal tissues is consistent with the concept that the function of D3 is to limit the exposure of fetal tissues to thyroid hormones.

Deiodination of the outer ring of T4 by D1 or D2 has been termed the activating pathway of T4 metabolism as it generates the most active thyroid hormone, T3. By this pathway, deiodinases play a critical role in maintaining tissue and cellular thyroid hormone levels of T3, which is then available to bind to hormone binding nuclear receptors and initiate thyroid hormone specific effects. Due to alterations in rates of local deiodination in tissues, thyroid hormone signaling can change irrespective of serum hormonal concentrations (3, 5). Deiodinases also modulate the tissue-specific

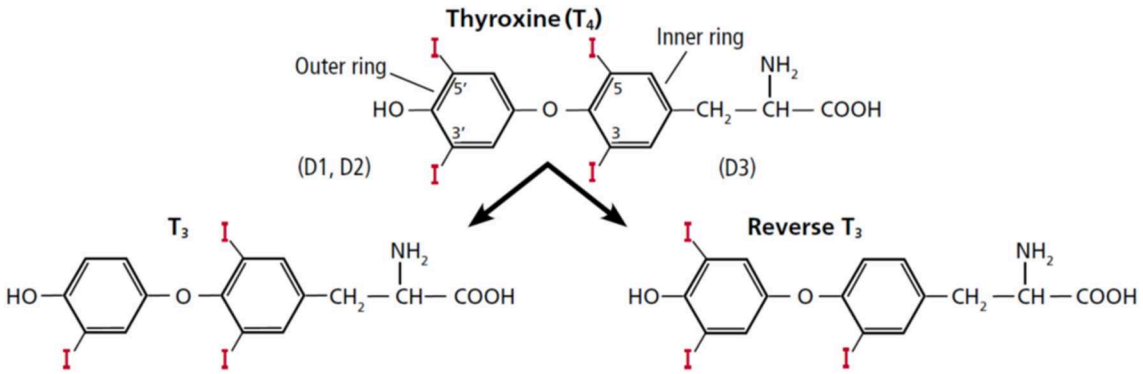

FIGURE 1 | Structure of thyroxine (T4) and the deiodinative cascade to T3 and reverse T3 (rT3). This figure was obtained with permission from Gomes-Lima and Burman (4). Copyright © 2018 The Cleveland Clinic Foundation. All rights reserved.

TABLE 1 | Tissue distribution of deiodinases.

Deiodinase	Site of action	Resulting action	Tissue distribution in humans
D1	Inner and outer ring of T4	Activating: generates preferably T3	Liver, kidneys, thyroid, and pituitary.
D2	Outer ring of T4	Activating: generates T3	CNS, pituitary, brown adipose tissue, thyroid, placenta, skeletal muscle, and heart.
D3	Inner ring of T4	Inactivating: generates rT3	Hemangiomas, fetal liver, placenta and in fetal tissues; skin and CNS.

concentrations of T3 in response to iodine deficiency, hyperthyroidism, and hypothyroidism (3). Thus, during iodine deficiency or hypothyroidism, tissues that express D2, especially the brain, increase the activity of this enzyme to increase local conversion of T4 to T3. In contrast, D1 overexpression in hyperthyroidism contributes to a relative excess of T3 production, while D3 up-regulation in the brain protects the CNS from excessive amounts of thyroid hormone (3).

Although rT3 is not widely expressed in adult tissues, it can be re-expressed in several pathophysiological conditions, such as cancer, starvation, cardiac hypertrophy, myocardial infarction, chronic inflammation, and critical illness (5). In these critical conditions, where a reduction of the metabolism and a reduced T3 level is physiologically desirable, the conversion of T4 to T3 is reduced, while the conversion to rT3 is increased. This is the basis of the alterations in thyroid hormone levels in the euthyroid sick syndrome or non-thyroidal illness syndrome (NTIS) (6). In general terms, this syndrome is characterized by normal or even decreased serum TSH levels in the presence of low serum T3 levels and serum levels of free T4 that may be normal, increased, or decreased. Since thyroid function tests are abnormal but inconsistent with hypothyroidism, it is frequently challenging to decide whether these patients need immediate thyroid replacement therapy. Further confounding the assessment of thyroid function of these patients is the associated frequent administration of drugs such as glucocorticoids, heparin, and dopamine that also influence thyroid hormone economy (6, 7).

Our understanding of the deiodinative pathways for T4 was accelerated by the development of a specific radioimmunoassay for rT3 (8). Reverse T3 was identified in the serum of normal individuals, in patients with hypothyroidism, hyperthyroidism and athyreotic patients being treated with different doses of levothyroxine (**Figure 2**) (9). It soon became apparent that the reciprocal changes in serum T3 and rT3 in patients with severe non-thyroidal diseases represented homeostatic attempts to conserve energy (10). However, rT3 has not proved reliable in differentiating euthyroid sick syndrome and hypothyroidism in critically ill patients. One retrospective study demonstrated that variations in rT3 levels may be associated with a variety of thyroid states (11). Therefore, diagnosis of thyroid status and subsequent management of patients with critical illness should be based upon the combined estimation of TSH, free T4 and total T3 serum levels together with clinical parameters and close follow-up of the evolving clinical and laboratory picture (6, 11).

CLINICAL UTILITY OF REVERSE T3

The early belief that rT3 measurement could be a useful laboratory marker of the euthyroid sick syndrome prompted many physicians to request this assay for patients in the critical care setting. But it soon became clear that in such situations, rT3 levels must be taken into context with other thyroid function tests, namely TSH, free T4, and total T3. Unfortunately, use of the rT3 assay has not clarified the interpretation of thyroid function tests and thyroid status (11).

Of course, the overwhelming majority of patients with a history of thyroid dysfunction are not managed in the critical care setting but in the office. However, even in the outpatient setting, a few clinical situations can present as mild forms of the euthyroid sick syndrome. During fasting or relative caloric deprivation, for instance, serum T3 is decreased as a homeostatic response to conserve energy and protein. A simultaneous increase in rT3

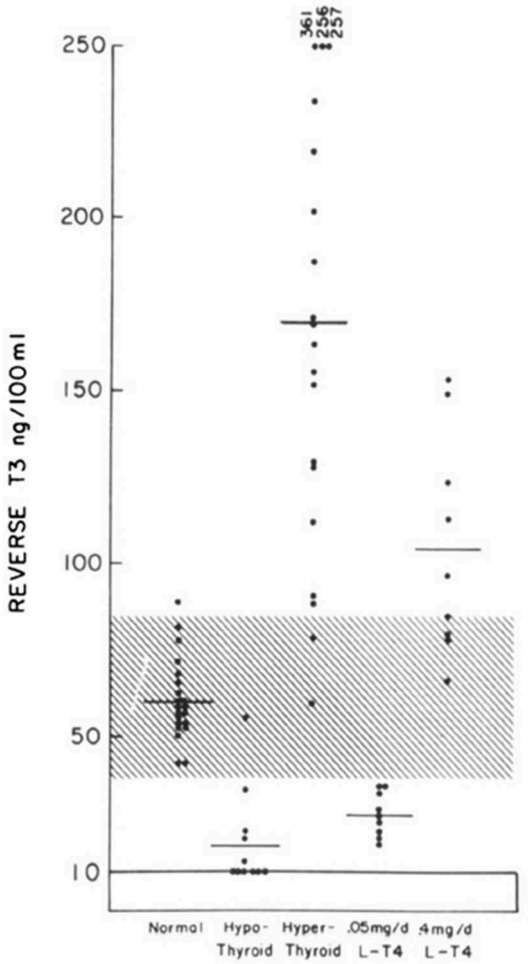

FIGURE 2 | Individual values of serum reverse T3 levels in normal, hypothyroid, and hyperthyroid subjects and in athyreotic patients who have been administered 50 mcg of LT4 and 400 mcg of LT4 daily [This figure was obtained with permission from Burman et al. (9)].

occurs in the first 2 weeks after caloric restriction, followed by normalization. Free T4 levels may be transiently increased or normal, while TSH levels may be normal or decreased (12, 13). Recognition of these laboratory abnormalities is important due to the increasingly popular use of fad diets that may include periods of fasting and deprivation of specific nutrients. Interpretation of the results of rT3 measurements in these patients should be approached with caution.

Before considering the possible use of rT3 to monitor patients on combined T4/T3 therapy vs. T4 treatment alone we should briefly review the apparent justification for such combined drug therapy. Most physicians caring for hypothyroid patients on T4 monotherapy see a significant subset of subjects who still complain of symptoms suggestive of thyroid hormone insufficiency in spite of TSH levels within the reference range. The argument made is that these patients suffer from insufficient T3 generation from T4. To attempt to generate T3 levels equivalent to those seen with thyroidal secretion of T3, the potential role and efficacy of combination T4/T3 treatment has been assessed. Having a blood test like rT3 to successfully address appropriate dosing of a T4/T3 combination agent could allow clinicians to more effectively treat patients with primary hypothyroidism.

The rationale for a need for combined T4/T3 therapy is based in large part on the discovery of polymorphisms in the deiodinase genes resulting in altered set points of feedback regulation of TSH. One common Thr92Ala polymorphism has been associated with insulin resistance, obesity, hypertension and importantly, altered responses to T4 replacement therapy that predicted the need for a higher T4 dosage, although only in thyroidectomized subjects but not in patients with autoimmune hypothyroidism (14–16). Similarly, patients with a rarer CC genotype of the rs225014 polymorphism in the deiodinase 2 gene showed a greater degree of improvement on T4/T3 therapy than on T4 monotherapy (17). Thus, while endocrinologists traditionally rely on TSH levels and TSH "normalization" during L-T4 therapy to reflect euthyroidism in all tissues, these deiodinase polymorphism studies shed some doubt on the validity of this practice for all patients in view of some clinical evidence supporting a role for combination T4/T3 treatment. These observations bring us back to the question as to whether monitoring another parameter of T4 metabolism, i.e., rT3, could better assess thyroid status by allowing us to target the optimal physiologic T4/T3 ratio. The rationale for T4/T3 combination treatment is based on the premise of low activity of D2 in selected patients, which does not necessarily imply higher D3 activity. Therefore, the rationale for the use of rT3 to monitor combination therapy would appear somewhat tenuous. Some studies comparing physiologic effects of combination T3/T4 treatment to L-T4 monotherapy have evaluated other parameters reflective of thyroid status such as serum SHBG or markers of bone turnover (18) but a practical and useful marker has not been identified.

So, can we justify the use of rT3 to guide levothyroxine therapy or levothyroxine (LT4) + liothyronine (LT3) combination therapy in hypothyroidism? There does not appear to be any rationale to measure rT3 to initiate or adjust levothyroxine therapy, and traditionally the best test for these purposes has been TSH measurement. Treatment decisions based on rT3 levels may lead to the use of excessive doses of levothyroxine, resulting in a state of subclinical or even overt hyperthyroidism (4). Moreover, rT3 assays are expensive and not widely available. They may be difficult to interpret depending on the assay used with the more reliable tests employing liquid chromatography/tandem mass spectrometry (LC-MS/MS). For patients who have elected combination therapy, it remains controversial as to what are the best biochemical parameters to monitor therapy. Potential targets include FT4, total T3, FT3, and FT4/FT3 ratio (19). The European Thyroid Association recommends monitoring thyroid function tests before the morning medications, aiming at normal TSH, FT4, FT3, and FT4/FT3 ratio (20). Yet the notable inaccuracy of FT3 assays requires precaution when interpreting serial laboratory results (19, 20). Unfortunately therefore, at the

present time there are no data that support for or against the use of rT3 to monitor LT4 + LT3 combination therapy.

It should be mentioned that there is one rare clinical entity for which the measurement of rT3 is essential to determine the correct diagnosis: in consumptive hypothyroidism. This is a rare form of hypothyroidism identified in newborns with infantile hepatic hemangiomatosis (21). It must be considered in the differential diagnosis of congenital hypothyroidism that requires inappropriately higher doses than usual. In 2000, Huang et al. reported the first case of severe hypothyroidism in a child with infantile hemangiomas (22). Despite the use of high doses of prednisolone and levothyroxine, the infant died from systemic complications after embolization of multiple hemangiomas. In that patient, the high serum levels of rT3 (413 ng/mL), followed by the demonstration of high expression of deiodinase type 3 (D3) in hemangioma tissue, were crucial to the diagnosis. This case report opened a new perspective in the understanding of the role of D3 in the euthyroid sick syndrome (23, 24). Understanding the molecular mechanisms that lead to the reactivation of D3 in illness is an important field of research. Several other cases of consumptive hypothyroidism have been reported so far; in adults they are even less common and have been associated with neoplasms (21, 25, 26).

CONCLUSION

Reverse T3 is physiologically relevant to thyroid economy. However, its clinical use as a biochemical parameter of thyroid function is very limited. Currently, no evidence supports the use of rT3 to monitor levothyroxine therapy, either given alone or in combination with liothyronine.

AUTHOR CONTRIBUTIONS

CG-L wrote the paper with input from LW and KB.

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frontiers in Endocrinology

HYPOTHESIS AND THEORY
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3,5-T2—A Janus-Faced Thyroid Hormone Metabolite Exerts Both Canonical T3-Mimetic Endocrine and Intracrine Hepatic Action

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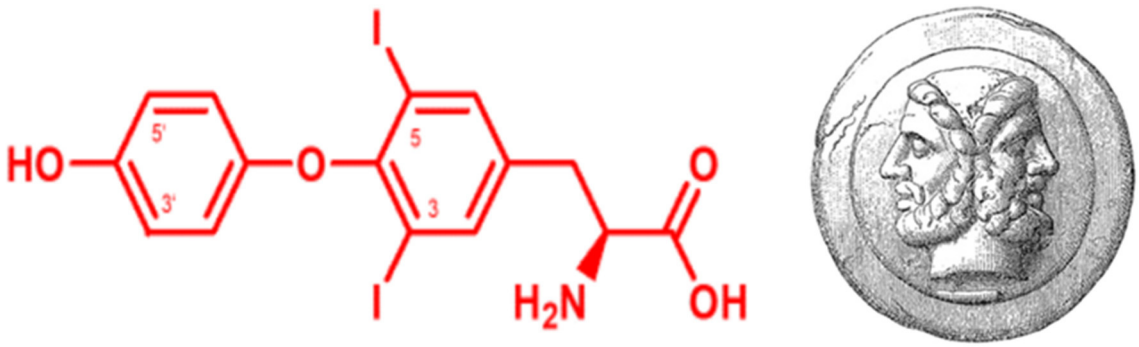
Over the last decades, thyroid hormone metabolites (THMs) received marked attention as it has been demonstrated that they are bioactive compounds. Their concentrations were determined by immunoassay or mass-spectrometry methods. Among those metabolites, 3,5-diiodothyronine (3,5-T2), occurs at low nanomolar concentrations in human serum, but might reach tissue concentrations similar to those of T4 and T3, at least based on data from rodent models. However, the immunoassay-based measurements in human sera revealed remarkable variations depending on antibodies used in the assays and thus need to be interpreted with caution. In clinical experimental approaches in euthyroid volunteers and hypothyroid patients using the immunoassay as the analytical tool no evidence of formation of 3,5-T2 from its putative precursors T4 or T3 was found, nor was any support found for the assumption that 3,5-T2 might represent a direct precursor for serum 3-T1-AM generated by combined deiodination and decarboxylation from 3,5-T2, as previously documented for mouse intestinal mucosa. We hypothesized that lowered endogenous production of 3,5-T2 in patients requiring T4 replacement therapy after thyroidectomy or for treatment of autoimmune thyroid disease, compared to production of 3,5-T2 in individuals with intact thyroid glands might contribute to the discontent seen in a subset of patients with this therapeutic regimen. So far, our observations do not support this assumption. However, the unexpected association between high serum 3,5-T2 and elevated urinary concentrations of metabolites related to coffee consumption requires further studies for an explanation. Elevated 3,5-T2 serum concentrations were found in several situations including impaired renal function, chronic dialysis, sepsis, non-survival in the ICU as well as post-operative atrial fibrillation (POAF) in studies using a monoclonal antibody-based chemoluminescence immunoassay. Pilot analysis of human sera using LC-linear-ion-trap-mass-spectrometry yielded 3,5-T2 concentrations below the limit of quantification in the majority of cases, thus the divergent results of both methods need to be reconciliated by further studies. Although positive anti-steatotic effects have been observed in rodent models, use of 3,5-T2 as a muscle

anabolic, slimming or fitness drug, easily obtained without medical prescription, must be advised against, considering its potency in suppressing the HPT axis and causing adverse cardiac side effects. 3,5-T2 escapes regular detection by commercially available clinical routine assays used for thyroid function tests, which may be seriously disrupted in individuals self-administering 3,5-T2 obtained over-the counter or from other sources.

Keywords: thyroid hormone, 3,5-diiodothyronine, hypothyroidism, metabolome, anti-steatotic action, coffee metabolites, chemoluminescence immunoassay, deiodinase



GRAPHICAL ABSTRACT | Structural formula of the "Janus-faced" THM 3,5-diiodo-L-thyronine (left), which has the same 3,5-iodine substitution pattern as its putative precursors L-T4 and L-T3 at the tyrosyl-ring, but lacks the iodine substitution in 3′-position of the phenolic ring which is essential for binding classical T3 -receptors and conveying canonical and non-canonical thyromimetic effects. The roman god Janus symbolized "duality" and "jani" were ceremonial gateways in ancient Rome typically used for symbolically auspicious entrances or exits. Janus-face (right) source: This image comes from the 4th edition of Meyers Konversationslexikon (1885–90). The copyrights have expired and this image is in the public domain. Wikimedia, Meyers_b9_s0153_b1.png.

HYPOTHESES AND THEORY

a. 3,5-T2 is an endogenous metabolite of thyroid hormones T4 and T3
b. 3,5-T2 might represent the precursor of 3-iodothyronamine
c. 3,5-T2 acts like T3 via canonical activation of T3 receptors albeit with lower potency
d. 3,5-T2 exerts actions distinct from those of thyromimetically active T3
   i) via mitochondrial targets
   ii) by its intrahepatic accumulation
   iii) by its intracrine mode of action
e. 3,5-T2 formation and action might be altered in patients on T4 replacement therapy and causes adverse effects if abused.

INTRODUCTION

Endogenous Thyroid Hormones and Their Metabolites

A century of thyroxine research has led to the commonly held opinion in the thyroid hormone community that 3,3′,5,5′-tetraiodo-L-thyronine (L-Thyroxine, L-T4), which is solely produced by the thyroid gland, serves as a prohormone while T3 (3,3′5-triiodo-L-thyronine), in part secreted by the thyroid gland, is mainly generated in various extra-thyroidal tissues by either type 1 or type 2 deiodinase (DIO1, DIO2) selenoenzymes (1–3). T3 exerts the majority of known thyroid hormone (TH) effects at the tissue and cellular level, including hypothalamic and pituitary negative feedback regulation of the hypothalamus-pituitary-thyroid-periphery (HPTP) axis (4–6). Over the last years, some evidence has been presented that T4 also binds to cell membrane-located αvβ3 integrin receptors which exert rapid signaling via various kinase and intracellular pathways, especially in tumor cells and stem cells (7, 8). The T4 metabolite Tetrac, a deaminated side chain metabolite, present in human serum at concentrations similar to those of T3 (3, 9) antagonizes such T4 (and also T3) actions at the integrin receptor signaling. However, whether this has physiological relevance beyond these pharmacological approaches, mainly tested so far in cancer or stem cells, remains to be demonstrated. A further metabolite of T4 found in human serum, 3,3′,5′-triiodothyronine (reverse T3, rT3) has been studied over the last four decades because its production by the type 3 deiodinase (DIO3), also a selenoprotein, is increased under conditions when T3 production by DIO1 is impaired (3, 9) as well as during development in many tissues, when DIO2 activity is decreased (10). As rT3 is also degraded by DIO1, such conditions of high rT3 and low T3 (and T4) serum concentrations, summarized under the term "non-thyroidal critical illness" or "low T3 syndrome," have caught the attention of clinicians attempting to use this constellation

as a diagnostic or predictive readout. Up to now, no clear demonstration of rT3 function has been observed apart from its role during glial cell-mediated neuronal guidance in mammalian brain development (11). The initial hypothesis that rT3 might act as potent inhibitor of DIO1 or DIO2 during T3 formation (12, 13) could not be supported by *in vivo* experiments due to its short half-life and insufficient local concentrations (14). These observations did not support the hypothesis of rT3 acting as an autonomous regulator of extrathyroidal T3 formation under (patho-)physiological conditions.

3,5-T2 Is a Further Endogenous TH Metabolite With Thyromimetic Potency

The TH metabolite 3,5-T2, possibly formed from its precursor T3 (**Figure 1**), has recently attracted great interest for several reasons (3, 9, 15). 3,5-T2 has been considered the main biological active metabolite of T3, formed via further phenolic ring deiodination from T3 (**Figure 1**). The TH metabolite 3,5-T2 is found in blood and at even higher concentrations in several tissues. Various groups have demonstrated that 3,5-T2, in addition to its thyromimetic action at the classical T3 receptors at high concentrations, exerts rapid direct effects on mitochondria (6, 16–19), which might be beneficial in terms of stimulation of oxygen consumption, increased hepatic, and muscular lipid metabolism—all of these effects appear as potentially favorable in global attempts to combat steatosis in liver and other tissues.

Therefore, we intended to test several hypotheses using clinical experimental data, animal experiments, and cell culture approaches as well as recently developed novel analytical tools such as the sensitive chemoluminescence immunoassay (CLIA), highly specific for 3,5-T2 detection in human serum (20). We aimed to test the hypotheses that

i) 3,5-T2 is formed from T4 and T3 as its precursors via deiodinase reaction,
ii) 3,5-T2 circulates in human serum, and that various pathophysiological as well as experimental conditions lead to alterations in 3,5-T2 serum concentrations.
iii) Furthermore, that effects of administration of physiological and pharmacological doses of 3,5-T2 in mouse models will provide information on its potential thyromimetic activity as well as for its potentially beneficial antisteatotic action.
iv) Moreover, analyses using various *in vitro* cellular models might convey an insight into postulated mechanism(s) of action of 3,5-T2 at the level of gene expression and/or mitochondrial energy metabolism.
v) 3,5-T2 causes adverse effects if abused and/or overdosed.

This series of clinical, translational and basic science studies testing these options resulted in a complex picture of various actions of 3,5-T2. The outcome was variable depending on concentrations applied, experimental models used, and analytical tools applied so far. 3,5-T2 turned out to be an ambiguous, *Janus*-type endogenous thyroid hormone metabolite (THM) with beneficial and adverse biological effects. Analytical tools available, cannot yet dispel previously reported controversies regarding its baseline concentrations in human serum and the variations in concentration caused by pathophysiological states.

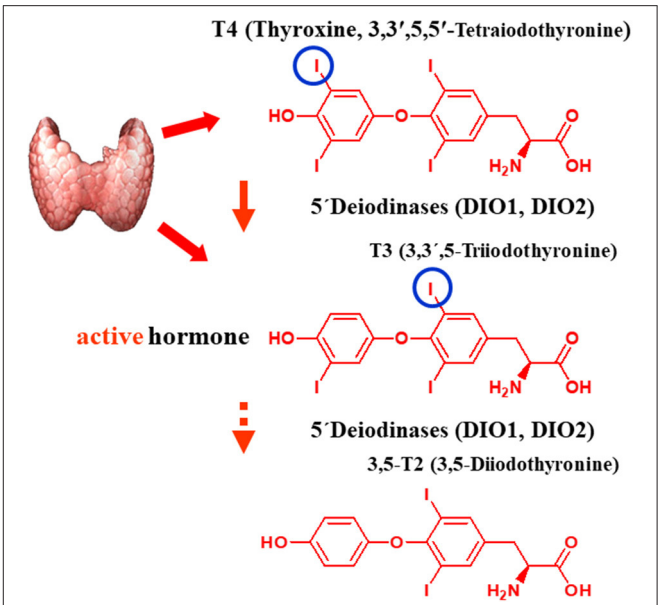


FIGURE 1 | Postulated pathway of biosynthesis of 3,5-T2 from its putative precursors T4 and T3. The figure shows the structural formulas of L-T4, the prohormone, synthetized, and secreted by the thyroid gland, and its 5′-deiodination product L-T3, which is secreted in part by the thyroid gland (ca. 80%) or generated in extrathyroidal tissues by the two selenoenzyme 5-deiodinase type 1 or type 2, which both remove the 5′-iodine atom of L-T4 in a reductive two-substrate reaction with a so far unknown physiological cofactor. Indirect evidence mainly from *in vivo* experiments in rodents suggests that 3,5-T2, a physiologically active endogenous thyroid hormone metabolite, is formed by a further 3′-deiodination reaction also catalyze by these (one of these) two deiodinase enzymes.

Currently, the thyroid community and patients discuss why a fraction of patients on established L-T4 replacement therapy, due to thyroidectomy or autoimmune thyroid disease, are subjectively discontent with this therapeutic regimen compared to individuals with intact thyroid, albeit their thyroid function test are in the reference range. We hypothesize that endogenous production of 3,5-T2 in some patients dependent on oral L-T4 might be less than that in individuals whose intact thyroid is the source of T4, and thus an inadequate 3,5-T2 concentration might contribute to this discontent based on its distinct thyromimetic activity compared to T3.

3,5-T2 CONCENTRATIONS IN HUMAN SERUM

Immunoassays for 3,5-T2 Using Polyclonal Antisera Reveal Divergent Concentrations

Following the detection of the thyromimetically active T3 in human serum and its production from T4 in athyreotic humans supplemented with L-thyroxine (21, 22), attempts were also made to quantify concentration of 3,5-T2 in human

TABLE 1 | Serum concentrations reported for 3,5-T2 and 3,3′-T2 over the last 4 decades using different analytical techniques (immunoassay formats, mass spectrometry).

References	Assay type	Serum 3,5-T2 (pM)	Serum 3,3′-T2 (pM)	Species
Richards et al. (23); Richards et al. (24); Richards et al. (25)	LC/MS-MS	<5	**41** ± 8	Human
Lehmphul et al. (20)	CLIA	**290** ± 10	–	Human
Gu et al. (26)	LC/MS-MS	–	**52** ± 16	Human
Jonklaas et al. (27)	LC/MS-MS	–	**12.2–28.2**	Human
Moreno et al. (28); Pinna et al. (29)	RIA	**4.6** ± 0.4	**68** ± 21	Rat
Pinna et al. (30)	RIA	**16.3** ± 6.4	–	Human
Engler and Burger (31)	RIA	**7.6** ± 3.4	–	Human
Jaedig and Faber (32)	RIA	**70**	**42**	Human
Kirkegaard et al. (33)	RIA	**105** ± 51	**36** ± 15	Human
Nishikawa et al. (34)	RIA	**196** ± 36	**67** ± 36	Human
Pangaro et al. (35)	RIA	**82** ± 4	–	Human
Maciel et al. (36)	RIA	**139** ± 67	–	Human
Meinhold and Schürnbrand (37)	RIA	**100**	–	Human

Bold values give mean serum concentrations reported by authors.

serum and tissues. Most of these studies were feasible after the development of specific radioimmunoassays, based on poly- or monoclonal antibodies in the Seventies of the last century, and supported extrathyroidal formation of 3,5-T2, which had been previously demonstrated using radioisotope-labeled TH in humans, experimental animal models, and *in vitro* systems [see recent reviews: (3, 9)]. Initially, various groups attempted to quantify 3,5-T2 under physiological and pharmacological conditions and in various disease states, mainly using radioimmunoassays based on specific polyclonal antibodies generated against 3,5-T2 conjugates in various animal models. The assays developed during the 1980s were sensitive enough to detect 3,5-T2 in unextracted serum samples directly, as summarized in **Table 1**. In contrast to rather precise and narrow concentration ranges detected for total T4, total T3, and total rT3 in human serum, the concentration ranges for 3,5-T2 showed remarkable differences between various assays used, exceeding more than one order of magnitude from 4 to almost 200 pM/L. This is in sharp contrast to concentrations reported for the other THM formed from either T3 via 5-deiodination at the tyrosyl- ring (3,3′-T2), or from rT3 generated by phenolic ring deiodinaton (3,3′-T2) or the rare T2 metabolite 3′,5′-T2 generated from rT3 via tyrosyl-ring deiodination. Reported serum concentrations covered a very narrow concentration range between 36 and 68 pM for 3,3′-T2 (see **Table 1**) (23–29, 32–34). Authors discussed this point, but no clear explanation or hypothesis was put forward to explain this peculiar observation atypical for the usually highly precise immunoassays for THM. The variable results might be explained by technical difficulties in reproducibly synthesizing and purifying tyrosyl-ring mono-labeled tracers required for these competitive immunoassays, as it turned out to be very difficult to avoid additional radioactive labeling of the phenolic ring of 3,5-T2, which would yield a T3-analog tracer. Only complex synthesis procedures different from the typical Chloramine T labeling protocols used for T3, rT3, or T4 yielded clean, radioactive tracers solely mono-labeled at the tyrosyl-ring (38). Thus, reports on serum 3,5-T2 concentrations determined by immunoassay methods need to be interpreted with caution.

A similar problem exists also with respect to the purity of 3,5-T2 standards used in these immunoassays, as commercial preparations available now and in the past frequently were significantly contaminated with T3 in concentrations high enough to provide interference by T3 serum concentrations, which are typically an order of magnitude higher than those of 3,5-T2 in the 3 nM concentration range.

A second difficulty still not clarified is the possibility of a distinct binding of 3,5-T2 to (yet unknown) serum distributor or transporter proteins, in comparison to T3 and T4 which are known to preferentially bind to the classical thyroid hormone distributor proteins thyroxine-binding globulin (TBG), transthyretin (TTR), and albumin. If residual binding to any serum distributor protein for 3,5-T2 would not be eliminated by incubation conditions or additives releasing 3,5-T2 from this binding, the assumptions of adequate equilibrium competition during incubation time and separation of free ligands from those bound to the antibodies would be compromised. Irrespective of these difficulties, various research groups set out to analyze 3,5-T2 concentrations in human serum in reference populations and individuals with altered thyroid function as well as disease states, but no uniform picture emerged and even observations of concentration changes in clinical hyperthyroidism vs. hypothyroidism were not uniform. Of interest was the observation that tissue levels of 3,5-T2, for example in the rat brain, were much higher than expected from serum concentrations and reached values in the range of those of T4 and T3, as clearly shown by Pinna et al. (29), who also observed changes in 3,5-T2 concentrations of various rat brain regions after administration of drugs used in neurology and psychiatry.

Similarly, a notable hepatic accumulation of 3,5-T2 was observed after administration of exogenous 3,5-T2 in a mouse model (39) suggesting a role as a tissue-resident "intracrine" hormone.

A Chemoluminescence Assay Based on Monoclonal Antibodies Detects 3,5-T2 Serum Concentrations Not Directly Related to Classical Parameters of Thyroid Hormone Status

We set out to develop a monoclonal antibody-based CLIA, which allows detection of 3,5-T2 in human serum at low concentrations and under various pathophysiological conditions. **Figure 2** illustrates the assay principle used (20, 40). Monoclonal antibodies recognizing 3,5-T2 with high specificity and very low cross-reactivity to other THM present in human serum were incubated in a micro-titer plate assay format, wells were coated with goat anti-mouse Fc antibody, and serum containing 3,5-T2. After incubation and various washing steps, horseradish peroxidase labeled 3,5-T2 competed with serum 3,5-T2 for binding to the anti-3,5-T2 monoclonal antibodies, and after equilibration of this competitive arrangement, chemiluminescence substrate was added to produce light and quantifying HRP labeled 3,5-T2 tracer vs. endogenous 3,5-T2. Using this assay, validated according to state-of-the-art technology with respect to assay stability, specificity, and reproducibility, we detected serum 3,5-T2 concentration in reference populations around 290 pM. Application of this immunoassay to analyze 3,5-T2 concentrations under various clinical conditions yielded unexpected results. First of all, 3,5-T2 concentrations in hypothyroid or hyperthyroid patients were not different from those of the reference population, while total T4 and T3 showed expected changes. We did not observe gender- or age-dependent variations, nor were concentrations correlated with BMI. However, thyroidectomized patients (after thyroid cancer diagnosis) showed slightly elevated serum concentrations compared to the healthy reference population (20), an observation to be confirmed by LC-LIT-MS/MS/MS (LC-LIT-MS^3) analysis. Patients with post-operative atrial fibrillation (POAF) also had higher 3,5-T2 concentration than a similar group of cardiology patients without POAF (41). Of interest was the observation that, in a group of intensive care unit (ICU) patients, non-survivors had significantly higher 3,5-T2 concentrations than those who survived (42). This was unexpected considering the low T3 concentrations in those patients in context of the hypothesis that 3,5-T2 would be a direct product of T3 and potentially lowered under conditions of decreased DIO1 (or DIO2) activity. Even more surprising was the observation of remarkable changes of 3,5-T2 concentrations, which were increased by more than an order of magnitude in patients on pre-dialysis compared to post-dialysis, with demonstration of even lower concentrations in healthy controls (20). **Figure 3** indicates this remarkable concentration range and increase in 3,5-T2 concentrations, which, with respect to the amplitude observed, is much higher than reported for any of the changes in T4, T3, rT3, or 3,3′-T2 concentrations so far. This data and the observation in ICU patients would be compatible with the hypothesis that impaired renal elimination of 3,5-T2 leads to its accumulation in serum or, alternatively, impaired renal function might contribute to elevated 3,5-T2 production. Such hypotheses need to be tested in appropriate prospective studies. Still those observation based on CLIA analysis of serum concentrations of 3,5-T2 require confirmation by LC-LIT-MS^3 analysis.

During our attempts to establish a reference range for 3,5-T2 in a healthy population, we analyzed 3,5-T2 concentrations in a subset of the population-based Study of Health in

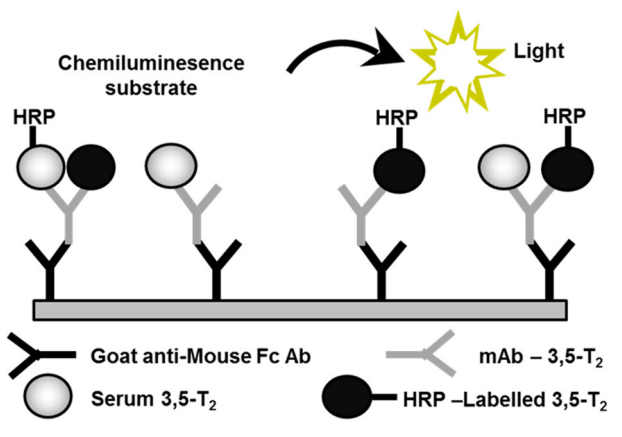


FIGURE 2 | Assay principle used for the development of a monoclonal antibody-based chemoluminescence immunoassay (CLIA). This method allows to detect 3,5-T2 in human serum at low concentrations and under various pathophysiological conditions (20, 40). HRP, horse-radish peroxidase; mAB, monoclonal antibody; Fc Ab, Fc-fragment of a goat antibody recognizing mouse mAb.

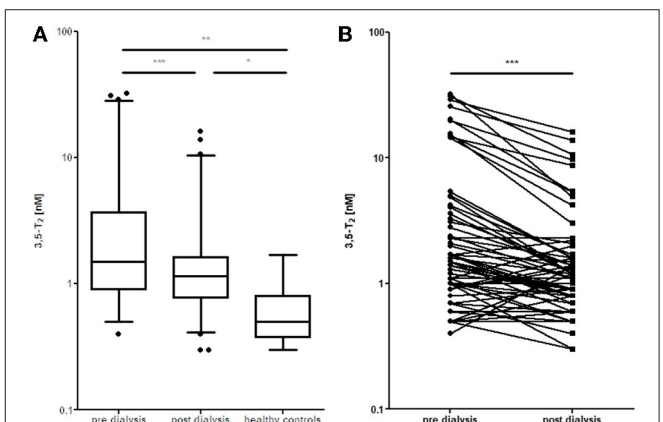


FIGURE 3 | Elevated 3,5-T2 concentrations in serum of dialysis patients. **(A)** Decrease in serum 3,5-T2 concentrations after dialysis, but still elevated compared to healthy controls (*$p < 0.05$; **$p < 0.01$; ***$P < 0.001$). **(B)** Comparison pre vs. post dialysis; paired *t*-test, Wilcoxon signed rank test (Gaussian approximation) (***$P < 0.001$) (43).

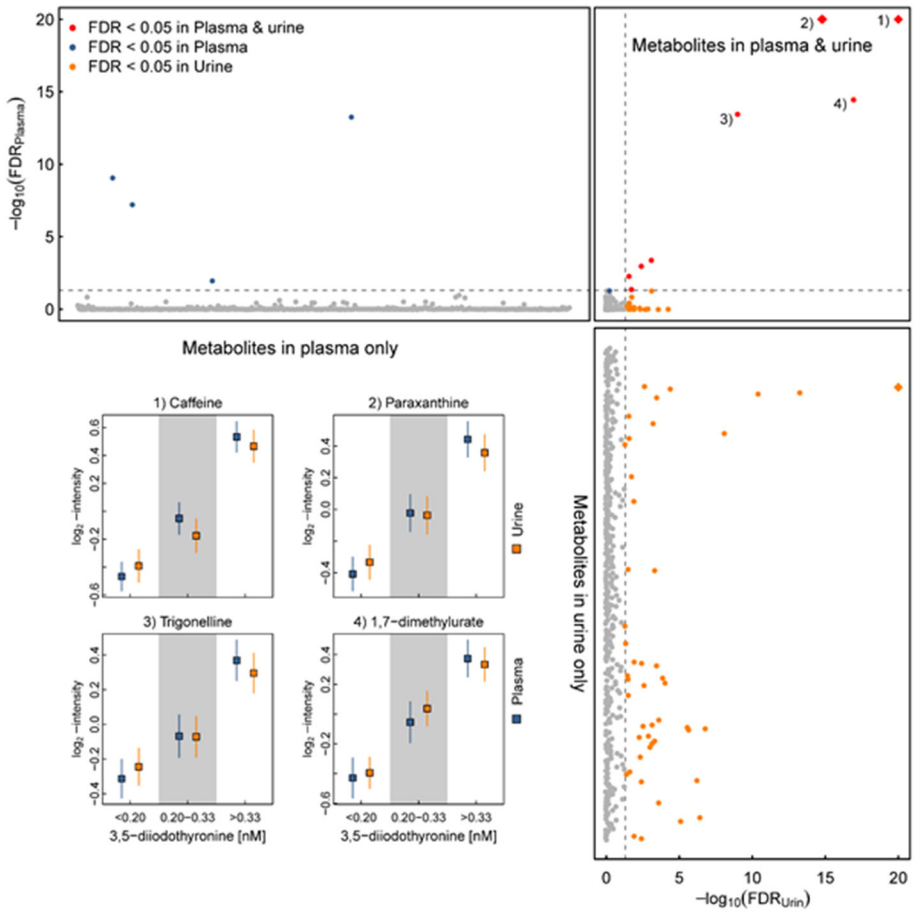


FIGURE 4 | Serum 3,5-T2 concentration and multifluid (urine and plasma) metabolomics in healthy individuals of the SHIP-Trend cohort. **(Upper)** *P*-values (y-axis) for plasma metabolites (x-axis) after correction for multiple testing (false discovery rate—FDR) comparing participants with low serum 3,5-diiodothyronine (3,5-T2) concentrations (<0.20 nM) with participants with high serum 3,5-T2 concentrations (>0.33 nM). Values have been transformed by $-\log_{10}$ to ease visualization of small values. **(Upper right)** Opposing FDR-values for the same comparison for metabolites measured in plasma (y-axis) and urine (x-axis). **(Left)** Similar to upper panel but showing the results for metabolites measured in urine only. Darker colors indicate metabolites meeting the threshold for statistical significance (FDR < 0.05) and red indicates metabolites consistently associated in both fluids. Inset: Predicted least-square means with 95%-CIs from linear regression models for four metabolites selected across the three approximately equally-sized groups of participants divided according to serum 3,5-T2 concentrations (44). All *p*-values were derived from a linear regression model treating serum 3,5-T2 concentrations as categorical exposure and metabolites as outcome, controlling for age, sex, waist circumference as well as serum thyroxine and thyrotropin concentrations. Blue—plasma and orange—urine.

Pomerania (SHIP-Trend) including 761 euthyroid participants. The analysis comprised associations with various anthropometric and clinically relevant parameters. Surprisingly, one third of the study population had 3,5-T2 serum concentrations determined by CLIA below the limit of quantification but above the limit of detection, yielding a median serum concentration of 0.24 nM with right-skewed distribution. Associations of the 3,5-T2 concentrations with various clinical chemistry parameters were moderate. Of special interest was, similar to the previous studies, the lack of a clear association with serum T4 and/or T3 concentrations (44, 45). In a follow-up study of the same cohort, 3,5-T2 serum concentrations were analyzed with respect to urine metabolites analyzed by ^{1}H-NMR spectroscopy (45–48). Again, very few individuals had remarkably elevated 3,5-T2 serum concentrations that might be suggestive of underlying disease, although health status could not be traced back due to the population-based study using anonymized sera. However, 3,5-T2 serum concentrations were remarkably positively associated with various urine metabolites. More detailed metabolome analyses revealed that these metabolites represent a "signature of coffee consumers." Among these urinary metabolites are trigonelline, pyroglutamate, and hippurate. This observation is the first one to link a THM to coffee consumption, and so far, no clear-cut hypothesis has been tested to challenge a potential causal relationship (45, 46). However, indirect evidence might support the assumption that hepatic accumulation of 3,5-T2 (see below) may alter hepatic (and/or renal) metabolism of components consumed with (caffeinated and decaffeinated) coffee and thus even might provide a link between beneficial antisteatotic effects ascribed to 3,5-T2 and also to coffee consumption (44–48) (**Figure 4**). **Table 2** summarizes the observations made in various pathophysiological states with respect to altered

3,5-T2 concentration in human serum, as determined by the monoclonal antibody-based CLIA.

BIOSYNTHESIS—3,5-T2 MAY NOT BE GENERATED DIRECTLY FROM ITS PUTATIVE PRECURSORS T4 AND/OR T3 IN HUMANS

The discovery and demonstration of sequential mono-deiodination pathways catalyzed by the three deiodinase isoenzymes using tetra, tri-, di-, and mono-iodinated iodothyronines as substrates has led to the assumption that 3,5-T2 formation *in vivo* occurs via 5′-deiodination of T3 catalyzed by either DIO1 or DIO2, while not much evidence was available that 3,5-T2 would be a direct secretion product from iodinated thyroglobulin. The existence of 3,5-T2 in thyroglobulin has not been clearly demonstrated (49, 50) and appears biochemically improbable considering that TPO catalyzes coupling of mono- and/or di-iodinated tyrosine residues to yield either T4 or T3, while no such coupling was observed between diiodotyrosine and iodine-free tyrosine in reports available. Formation of iodothyronines outside of the thyroid gland has not been reported in humans or mammalian organisms while various aquatic life forms synthesize T4 without presenting the highly evolved follicular structure of thyroid glands in vertebrates (51). Circumstantial evidence using the DIO1 inhibitor PTU in isolated rat mitochondria or perfused rat liver led to the assumption that 3,5-T2 is a logical deiodinase product of T3 (16, 52), but various attempts to demonstrate such a reaction *in vitro* failed to support this hypothesis. In contrast, *in vivo* data from experimental animal models and human studies, where PTU has been administered, resulted in lower 3,5-T2 concentrations compared to appropriate controls. However, these studies were also based on immunoassay serum analytics.

In the light of marked variations of serum concentrations of 3,5-T2 in humans as determined by the monoclonal antibody-based CLIA, we set out to test whether 3,5-T2 is formed *in vivo* in humans if T4 and/or T3 were administered. Several experimental paradigms were tested for acute or chronic 3,5-T2 formation from T4 and/or T3. **Figure 5A** shows the experimental approaches used by Jonklaas et al. (53), who administered a single dose of T3 (50 μg) to 12 euthyroid individuals and sampled serum over 72 h. Assuming T3 as precursor of 3,5-T2, one would expect an increase of 3,5-T2 concentration after T3 administration. In a second paradigm (**Figure 5B**) (54), T3 was administered at concentrations between 30 and 60 μg to hypothyroid patients, who at baseline were substituted with T4. Their T4 was discontinued and they were instead treated with a daily dose of 15 μg of T3. Subsequently the T3 dose was increased by 15, 30, or 45 μg to replace T4 over a period of 4 weeks aiming for non-suppressed TSH. Blood samples were drawn at baseline and then weekly during T3 treatment. Blood samples were also drawn hourly for 8 h after the final T3 dose was given (**Figure 5B**). In a third paradigm, we determined 3,5-T2 serum concentrations (data not shown) in 10 thyroidectomized patients who were treated with L-T4 or L-T3 to target their serum TSH to the reference range of 0.5–1.5 mU/L for at least 30 days, in order to test whether T3 and T4 can lead to similar TSH concentrations in the reference range (55). In the fourth study, 3,5-T2 concentrations were determined (data also not shown) in sera of volunteers receiving 100 μg of T3 sulfate, an endogenous THM, which might act as reservoir or precursor to rapidly yield T3 liberated by ubiquitous sulfatase enzymes (56). T3 sulfate is formed during enterohepatic circulation of TH, and 3,3′-T2 sulfate is a metabolite generated by the fetus and transferred to maternal circulation during pregnancy (57, 58). Again, rapid formation of T3 from T3 sulfate might lead to elevated production of 3,5-T2 if T3 and/or T3 sulfate would be precursors of 3,5-T2. While analysis of T3 in serum for all four paradigms revealed the expected kinetic profiles and TSH responses (53, 55, 56), the 3,5-T2 concentration profiles found (**Figure 5C**) were unexpected. In all of the above mentioned four paradigms, irrespective of T4, T3, or T3 sulfate administration, significant changes of 3,5-T2 serum concentration as determined by mAB-CLIA were not observed [and also no changes in concentrations of 3-T1AM, also determined by monoclonal antibody CLIA (59), were found]. 3,5-T2 had been postulated as a potential precursor of the biogenic amine 3-iodo-thyronamine (3-T1AM), possibly enzymatically generated from 3,5-T2 by a combination of deiodination and decarboxylation (60, 61).

Together, these studies might indicate that 3,5-T2 is not directly generated from serum T3 and its serum profile does not mimic the transient increases observed for T3 after its exogenous administration in humans. Several interpretations can be put forward to explain this situation: (i) 3,5-T2 is not a direct product of T3, or (ii) increases in T3 or T3-sulfate concentration in serum are not reflected by corresponding coincident or delayed 3,5-T2 concentration profiles, (iii) but 3,5-T2 might be formed intracellularly in

TABLE 2 | Summary of serum 3,5-T2 concentrations in humans observed under physiological (a) and pathophysiological (b) conditions.

a) Physiological conditions:

- Serum 3,5-T2 and 3T1AM concentrations as analyzed by specific mAb-based CLIA do not mirror the dynamics of T3 (or T4) after substitution with T4, T3, or T3-sulfate in healthy individuals or hypothyroid patients
- Inter-individual differences in 3,5-T2 and 3T1AM serum concentrations
- Remarkably stable individual 3,5-T2 and 3T1AM serum concentrations
- Discrepancy between 3,5-T2 serum concentrations determined by CLIA and LC-MS/MS
- No clear correlation to TSH, T4, and T3 concentrations
- No evidence from these studies supporting the postulated metabolic pathway in humans: T4→ T3→ 3,5-T2→ 3T1AM
- Serum 3,5-T2 concentrations are correlated to trigonelline, hippurate, and 3-aminoisobutyrate concentration in urine of healthy individuals ("coffee signature").

b) Elevated serum concentrations of 3,5-T2 in patients:

- Sepsis
- Non-survivors of ICU
- Postoperative atrial fibrillation (POAF)
- Impaired renal function
- Oral T4 supplementation.

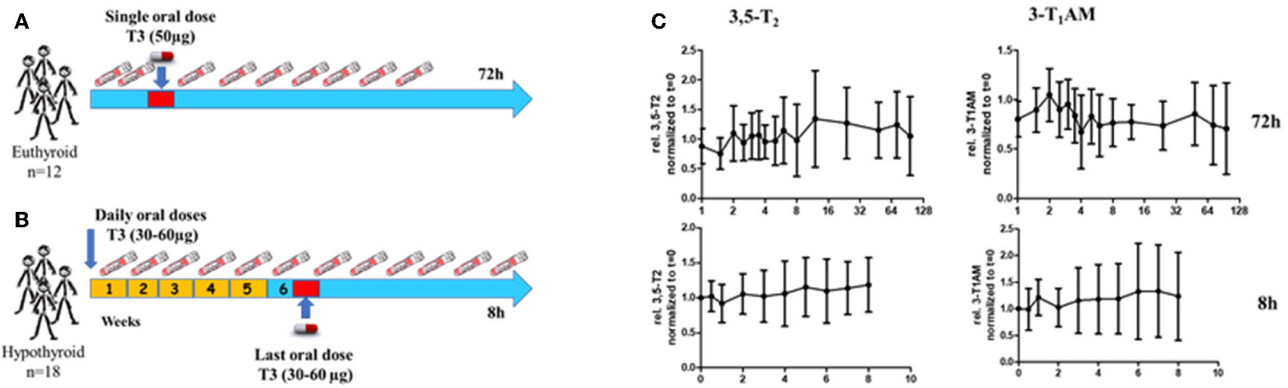


FIGURE 5 | Experimental paradigms to test whether 3,5-T2 is formed from T4/T3 in humans. **(A)** Twelve euthyroid healthy volunteers received a single oral dose of L-T3 (50 µg) and blood samples were drawn during the following 72 h (53). **(B)** Eighteen hypothyroid patients were switched from T4 to daily doses of 15 µg Liothyronine (T3) at week 1. Their daily T3 dose was then increased to 30 µg after week 2 and increased as necessary up to 60 µg T3 to maintain a normal serum TSH. Serum samples were drawn weekly and also for a kinetic study during the 8 h after the final T3 dose was administered in week 6 (54). **(C)** Serum concentrations (relative to individual concentrations at $t = 0$ h) for the two kinetic studies (see above) administering Liothyronine (T3) to euthyroid volunteers (upper panel) and hypothyroid patients (lower panel). Individual serum concentration data are provided in **Supplementary Data Sheet 1**. No clear increase was found in serum 3.5-T2 concentration in both paradigms and also no increase was observed for 3-T1AM, the postulated product generated from 3,5-T2 via combined deiodination and decarboxylation).

various tissues; (iv) the half-life of 3,5-T2 may be too short to lead to accumulation of this metabolite in human serum after T3, T3-sulfate[1], or T4 administration; (v) the monoclonal antibody-based immunoassay for 3,5-T2 may recognize also additional cross-reacting compounds not formed after *in vivo* application of T4, T3, or T3-sulfate. However, these observed patterns in sera of these four studies do contradict the above-mentioned observations that higher 3,5-T2 concentrations were observed in thyroidectomized patients on oral T4 replacement therapy (20, 62). **Figure 5C** shows the serum profiles of 3,5-T2 found after T3 administration in healthy controls and hypothyroid patients, respectively, in the two Jonklaas studies (53, 54). Assay results had to be normalized to time zero 3,5-T2 concentrations to generate the kinetic profile. Of note are quite distinct individual 3,5-T2 concentrations both in healthy individuals and hypothyroid patients that were not affected by T3 administration, with intra-individual 3,5-T2 serum concentrations that remained remarkably stable during several hours and days of sampling time (**Figure 6**).

3,5-T2 SERUM CONCENTRATIONS ARE REMARKABLY STABLE AND DISTINCT BETWEEN INDIVIDUALS

3,5-T2 concentrations in individuals exhibit remarkable stability over sampling time. **Figure 6** illustrates distribution of 3,5-T2 concentrations in various individuals over sampling time for several hours and days. **Figure 5C** represents the analysis of 3,5-T2 concentrations in healthy individuals ($n = 12$), to whom a single dose of 50 µg T3 was administered, and blood samples were taken regularly over 72 h (53). Most individuals exhibit very narrow concentration ranges with small variations over sampling time while individuals differ among each other in their 3,5-T2 concentration in serum. **Figure 5C** also shows similarly analyzed 3,5-T2 concentrations in 18 hypothyroid patients, who received T3 doses between 30 and 60 µg total, following discontinuation of T4 treatment (54). T3 was dosed to bring TSH concentrations into the euthyroid reference range. Compared to healthy individuals, inter-individual differences between 3,5-T2 concentrations were more pronounced; nevertheless, each individual again showed rather stable 3,5-T2 concentrations over time, considering that, as illustrated above in **Figure 6**, no significant changes in 3,5-T2 concentrations were observed after T3 administration. Similar patterns of rather stable individual 3,5-T2 concentrations were observed in a drug study on volunteers, who underwent food withdrawal and refeeding protocols while treated with an experimental drug (J.K. et al., unpublished data). But again, explicit inter-individual differences were observed between the volunteers, while 3,5-T2 concentrations remained fairly constant in each individual over several circadian phases, food withdrawal and refeeding cycles, and experimental drug administration. Only one individual was exceptional in exhibiting rather pronounced variations of 3,5-T2 for unknown reasons (unpublished data, van Vliet and Köhrle). Unfortunately, no residual, sufficiently large serum sample volumes (additional 200 µL) were available to repeat those studies using the recently developed new LC-LIT-MS[3] analysis of THM (25).

Taken together, the determination of 3,5-T2 concentrations by mAb-based CLIA in experimental human studies involving administration of T4, T3 alone, as well as T3 sulfate, or an experimental drug did not provide evidence that the postulated

[1]Data are not shown for the 3,5-T2 serum profile after administration of T3 sulfate or T4/T3 combination therapy.

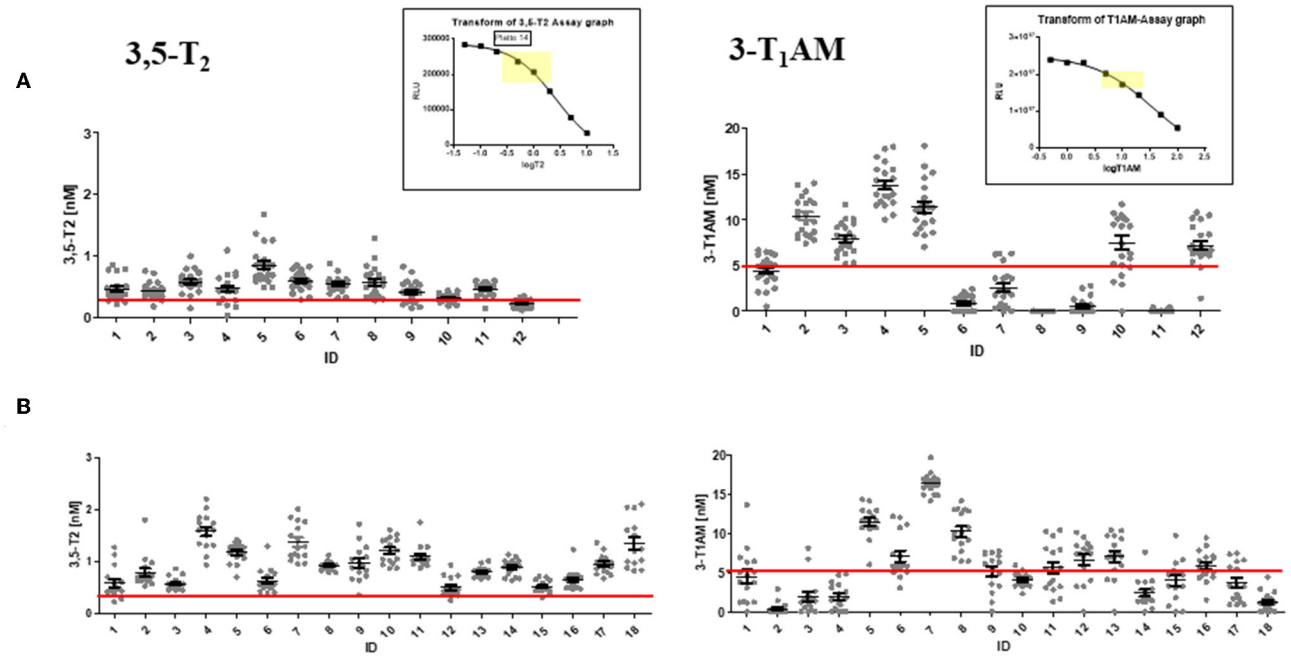


FIGURE 6 | Remarkably stable individual 3,5-T2 serum concentrations during several hours and days of sampling time but significant inter-individual differences. Graphs show absolute serum concentrations of 3,5-T2 (left) and 3-T1AM (right) for each individual receiving Liothyronine in the two kinetic studies presented in **Figures 5A,B**. Upper panel **(A)**: 12 euthyroid volunteers with blood drawn over 72 h; lower panel **(B)**: 18 hypothyroid patients with blood drawn over 8 h. Inter-individual differences for 3-T1AM serum concentrations seem to show higher variations than those for 3,5-T2, and variations over time might be more pronounced than those of 3,5-T2. Individual serum concentration data are provided in **Supplementary Data Sheet 2**. Insets show typical standard curve for 3,5-T2 and 3-T1AM CLIA standard curves, with reference range marked in yellow. The red line represents the preliminary reference concentration for healthy controls.

metabolic pathway of T4 → T3 → 3,5-T2 deiodination could be supported in analyses after short or medium-term interventions in humans. Thus, it remains to be studied whether 3′(5′)-deiodination of T3 to 3,5-T2 occurs in any human tissues and can be measured in serum in healthy volunteer individuals or hypothyroid patients requiring TH substitution.

ALTERNATIVE LC-MS-BASED APPROACHES ARE NEEDED TO DETERMINE CONCENTRATION OF THYROID HORMONES AND THEIR METABOLITES IN HUMAN SERUM

Over the last years, the discovery of various THM with either lower iodination grade or modifications at the functional residues of iodothyronines, that is the 4′-hydroxy group conjugation or modification of the alanine side chain, and subsequent studies on the potential biological relevance of various THM (see **Graphical Abstract**) has led to various attempts to quantify the whole spectrum of iodinated and iodine-free metabolites ["the thyronome" profile; (3)] using liquid chromatography—tandem mass spectrometry (LC-MS/MS) techniques [for a recent review see (23)]. Initial attempts to quantify T4 and its metabolites by GC-MS did not gain acceptance for clinical diagnostics. The MS-based analysis of THM is faced with several challenges: (i) first of all, should total and/or free concentrations be determined in whole blood, serum, plasma, or other body fluids like saliva, cerebrospinal fluid, or urine? (ii) How can THM be quantitatively analyzed considering their extremely high affinity binding to various distributor proteins contained in plasma, such as TBG, TTR, albumin, or APO-lipoprotein B100? (iii) Which chromatographic procedure faithfully will separate isobaric tri-, di-, or mono-iodinated structural isomers of T4 metabolites? (iv) Is the sensitivity of combined LC-MS sufficiently high to quantify in a single run nanomolar total T4 and T3 concentrations and low picomolar concentrations of mono- or di-iodinated THMs? (v) Which influence will matrix composition of plasma or serum from healthy individuals vs. sick patients exert, and can this be adequately standardized to accurately quantify the concentrations of the THM spectrum in clinical practice?

Appreciating the major progress made during the last two decades in mass spectrometry-based analytics of steroid hormones (63–66), several groups attempted to draw level with those laboratories in the TH community also. However, various practical and technical issues related to above-mentioned challenges of TH physiology and pathophysiology hampered rapid progress and success in quantification of total and free THM concentrations. While meanwhile several laboratories developed procedures to accurately determine total T4 and total T3 in human plasma or serum and largely agree on

concentrations ranges (25, 67–73), this consensus has not been achieved yet for most of the other TH metabolites, which occur at much lower concentrations. Analytical procedures typically work well with buffer solutions, *in vitro* reaction mixtures, as well as with samples of "simple" matrix composition, while reported concentrations in human or experimental animal serum, plasma, and tissue show wide variations and marked insufficiencies in terms of method validation, standardization, and quality assessment parameters [see (23, 24, 74)]. Most groups reporting data employed liquid-liquid or solid-phase extraction procedures with or without previous protein precipitation to enrich and purify THM profiles from matrix components. Nevertheless, sample recoveries, process efficiencies, and matrix effects were not convincing in most of the cases published, if reported at all. Although the teams used the principle of isotope dilution technique, as far as isotope-labeled standards for the individual thyronine metabolites are available, no uniform picture has evolved yet. It has become apparent that for accurate quantification of various THM, use of only one or two stable isotope-labeled internal TH standards is not sufficient due to the very distinct binding of THM, which are highly hydrophobic but still polar amino acid derivatives. This tight binding not only relates to their distribution proteins in blood, cells or tissues but especially also to surfaces of plastic material required during pre-analytical sample work and subsequent MS quantification. Recently, two groups reported for the first time 3,5-T2 serum concentrations determined by LC-MS. Lorenzini et al. (75), observed a mean concentration range for 3,5-T2 in human serum of 78 ± 9 pM, while our own results (25) indicated that most human sera analyzed for 3,5-T2 have lower than 5 pM 3,5-T2 concentrations, which is the lower limit of quantification in our method. The fact that Lorenzini et al. probably report higher LC-MS-based 3,5-T2 concentrations is due to the contamination of the $13^{C}9$-15^{N}-3,5-T2 internal standard, with "natural" unlabeled 3,5-T2. While we used very high dilution of this internal isotope labeled standard once we discovered this problem, Lorenzini et al. who used the same supplier for this material, probably included this "exogenously added 3,5-T2" in their analysis while adding much higher undiluted internal standard concentrations to their samples. A surprising outcome of both our own and the Lorenzini study, however, is that MS-based analysis of 3,5-T2 concentration in sera of healthy individuals is significantly lower than concentrations reported by our above-mentioned CLIA based on monoclonal antibodies. At this point, no explanation for the divergent results is possible yet, but needs to be considered during future analysis and further improvement of analytical methods for concentrations determination of 3,5-T2 in human and animal serum (25). The method used by Richards et al. involved a completely novel pre-analytical approach involving single step solid-phase delipidation, avoiding multiple use of plastic material during sample workup and employing silanized glass vessels during this procedure. This sample work-up was then combined with isotope dilution, LC-positive ion electron spray multiple reaction monitoring (MRM) linear ion trap LIT-MS^3, which allowed clear separation of isobaric tri- and di-iodinated iodothyronines and resulted in acceptable signal-noise ratios for the LIT-MS^3 scans leading to limits of quantification in the low picomolar range. Assay accuracies, precisions, and process efficiencies with quality assessment parameters were obtained which are requested by authorities for analytical procedures utilized in clinical diagnostics. Application of this procedure allows for simultaneous, pre-analytical extraction and mass-spectrometry quantification of T4, T3, rT3, 3,3′T2, and 3,5-T2 in one single run using 200 µl serum (or less, if the two di-iodinated thyroid hormones are not required for these analytics). Major work is needed to clarify the differences between mAb-based immunoassays and MS-based analytics. Previously, other groups already reported major deviations between immunoassay- and LC-MS-based methods, if T3, rT3, or 3,3′T2 were analyzed in human serum (27, 76, 77). Application of this new method for determination of free TH concentrations will require significantly more work on method development and quantification.

TABLE 3 | Ligand binding and cross reactivity of monoclonal antibodies used in 3,5-T2 and 3-T1AM CLIA as analyzed by immune extraction.

mAb	$^{2}H_4$-3-T_1AM*	$^{13}C_9$-^{15}N-3,5-T_2^*	3,5-T_2	T_3	rT_3	T_4	3-T_1-AM
(3-T_1-AM)	✓	▬	▬	▬	▬	▬	✓
(3,5-T_2)	▬	✓	✓	▬	▬	▬	▬

*Ligand binding specificity and cross reactivity with other thyroid hormones/metabolites was analyzed by immune extraction from native and spiked serum in parallel using monoclonal antibodies (mAb) employed in the CLIA immune assays. Extracts bound to the mAb were subsequently analyzed by LC-MS-MS. No significant cross reactivity for the analytes indicated was detectable. *Stable-isotopically-labeled THM used as internal standards in LC-MS/MS.*

Using the monoclonal antibodies employed in the CLIA methods, a series of immune extraction experiments from native or spiked human serum was performed to clarify the differences found in 3,5-T2 concentrations in human serum using either the CLIA or LC-MS method (unpublished work). Human serum aliquots were either used as prepared after blood drawing or after spiking with analytes of interest. Aliquots with known amounts of either 3,5-T2 or 3-T1AM sera were incubated for equilibration at room temperature for 1 h, then highly specific mAbs against 3,5-T2 (20) or 3-T1AM (59) were added in form of beads covalently bound to these antibodies, then sera were incubated overnight at 4°C, serum was removed by centrifugation, and antibody beads were recovered. Beads were washed several times, and bound THM were eluted with methanol and analyzed either by LC-MS/MS (25) or TOF-LC-MS. Evaluation of extracts from mAb beads by LC-MS analysis (25) revealed that the 3,5-T2 antibody efficiently extracted 3,5-T2 from serum as expected but did not bind and extract spiked 3-T1AM or other endogenous THM. In contrast, the mAb against 3-T1AM, also coupled to beads, extracted spiked 3-T1AM from serum, but no spiked 3,5-T2, no other THM and surprisingly not any "endogenous" 3-T1AM was found in the extracted eluates (**Table 3**) (J.K. et al., unpublished data). Careful analysis by TOF-LC-MS did not provide information on significant enrichment or extraction of any unknown serum components cross-reacting either with the 3,5-T2 or the 3-T1AM mAbs to a measurable extent. Thus, a carefully controlled immune-extraction approach

of native or THM spiked serum did not reveal, why 3,5-T2 concentrations determined by mAB-based CLIA were so much higher than those endogenous 3,5-T2 concentrations determined by LIT-MS[3] and the recently described one-step pre-analytical extraction method.

3,5-T2 MECHANISM OF ACTION INVOLVES CANONICAL TR SIGNALING AND RAPID DIRECT EFFECTS

3,5-T2 Action on Liver Is Accompanied by Potentially Adverse Effects on HPT-Axis and Heart

3,5-T2, in contrast to its structural isomers 3,3′-T2 and 3′,5′-T2, is an active ligand for the classical T3 receptors (TR), where it binds with relative high affinity (**Table 4**) and modulates transcriptional activity of TR, as indicated e.g., by rapid and powerful suppression of TSH *in vivo*, altered 3,5-T2 modulated gene expression *in vitro* and in several cellular models including anterior pituitary and liver (39, 81, 85–87). In addition to its action on TSH suppression in thyrotropes, 3,5-T2 also stimulates growth hormone production and secretion in somatotrophs (86). Apart from this work, elucidating thyromimetic effects similar to those exerted by T3 *in vivo* as well as in various experimental and cellular models, a group of researchers working with Goglia et al. over the last three decades presented evidence that 3,5-T2 might exert direct effects on mitochondria via interaction with proteins of the electron transport chain (e.g., cytochrome C oxidase) and with F0-ATPase [for review see (17)]. In addition to these actions, probably not mediated by mechanism related to the canonical TR nor its mitochondrial form, TRαp43 or TRαp22 (88), these authors claimed that 3,5-T2 might exert several beneficial actions on hepatic, muscular, and adipocyte tissues already at "low" concentrations. In their models apparently neither suppression of pituitary TSH production and secretion nor T3-typical adverse effects on the heart were observed at these doses used. Most of these studies were performed in severely hypothyroid, mostly rat models, and 3,5-T2 doses were administered acutely as well as chronically. Unfortunately, no information had been published on 3,5-T2 concentrations reached in the circulation or in those target tissues like liver, where beneficial effects such as antisteatotic activity and increased lipid metabolism were reported (15, 17, 28, 89, 90). The first evidence for rapid T3-independent effects of 3,5-T2 on mitochondrial activity and oxygen consumption was provided by Horst et al. (16), who observed rapid stimulation of oxygen consumption in isolated perfused livers, removed from hypothyroid rats. In their model, already low picomolar 3,5-T2 concentrations stimulated O_2 consumption within 90 min, while T4 and T3 reacted more slowly and their effect could be blocked by adding 1 μM PTU to the perfusion medium, which acts as an inhibitor of DIO1, assumed to be the enzyme responsible for 3,5-T2 production. These authors studied several TH analogs with respect to stimulation of oxygen consumption in their model, in part complemented by analyses of stimulation of alpha-glycerophosphate-dehydrogenase, which is a typical biomarker for TH action on mitochondria. Moreno et al. (90) performed further studies on the time course and mechanisms involved in rapid stimulation of resting metabolic rate caused by THM. They observed that 3,5-T2 (25 μg/100 g body weight) exerted rapid effects in acutely treated hypothyroid rats while T3 reacted more slowly but with longer lasting effects compared to 3,5-T2. The protein synthesis inhibitor actinomycin D inhibited the effects of T3 similarly to observations made by Horst et al. (16) who showed that cycloheximide could block the T3 effect in their perfused liver model. Subsequently, the Naples teams, with investigator Goglia, added more endpoints to demonstrate rapid 3,5-T2 action, such as increased glucose consumption in muscle, which might involve sirtuin signaling (91). Several pathways involved in mediation of 3,5-T2 effects were subsequently studied using gene expression, functional, and proteomic readouts, especially for rat liver and muscle (92, 93) while comparable control data on potentially adverse effects of 3,5-T2 concentrations resulting in changes of liver and muscle metabolism were not systematically presented.

Controversial with respect to this set of observations, several other teams reported that administration of 3,5-T2 in rodent models (rat and mice, regular or high-fat diet, euthyroid or hypothyroid) dose-dependently resulted in significant suppression of the HPT axis as reflected by decreased TSH serum concentrations and/or pituitary beta-TSH transcript levels (6, 39, 87, 94, 95). Furthermore, thyromimetic effects typical for high circulating T3 were observed in the heart as analyzed by gene expression and/or morphological alterations suspicious of remodeling and fibrosis. Typically, those changes were then also accompanied by altered expression of T3 responsive genes in liver and other tissues. There have also been observations that 3,5-T2 interferes with the function of pancreatic islets altering glucagon and insulin expression and secretion (91, 96). Thus, it remains quite controversial whether 3,5-T2 would be an important lead compound in the development of antisteatotic drugs lacking unwanted adverse side effects at other TH responsive target tissues and reactions. The Naples teams also reported a pilot volunteer study, where two of the involved researchers dose escalated 3,5-T2 administration

TABLE 4 | Relative potencies of thyroid hormone metabolites at thyroid hormone receptors expressed as fold concentration required to displace T3 from binding to TRβ1 which is set as 1 (in bold) (corresponding to a $K_d = 1 \times 10^{-10}$ nM).

Receptor	T4	T3	rT3	3,5-T2	Tetrac	Triac	References
TRα1	14	2–20	–	–	–	3.9	(78)
TRβ1	3–22	**1**	300	–	33	3.6	(79, 80)
TRβ2	–	32	–	40–500	–	4.2	(81, 82)
$pTR\alpha_{28}$	–	3	–	–	–	–	(83)
Integrin αvβ3	3	–	–	–	–	–	(84)

Published experimental data were obtained using various types of TH receptor preparations, Scatchard analyses or competition assays, resulting in IC-50 values. No systematic comparison was made using recombinantly expressed receptor under comparable binding conditions, ad different tissues, and species were used in competition experiments. Thus, only rough ranges are compiled here due to the limited comparability of experimental models.

on themselves without observing unwanted side effects but reported on weight loss (97). This observation could not be supported by a randomized, placebo-controlled, double-blind, 4-week trial on male cardio-metabolic patients, who received a 50 mg daily dose of TRC-150094 (98). This is a 3,5-T2 mimetic agent previously shown to ameliorate metabolic risks in a high-fat diet obese rat animal model, where oxidative metabolism was significantly stimulated both in liver and skeletal muscle, and several metabolic pathways such as nitrogen, amino acid and sugar metabolism were shown to be altered using, among others, proteome analysis (99). Different from this promising animal experiment, the clinical trial provided no evidence for increased insulin sensitivity, changes in plasma free fatty acids, or intra-hepatic triglyceride content after administration of this agent (98). No further studies are available on this agent in rodents or humans.

3,5-T2 Accumulates in Tissues and Might Act by Intracrine Mechanisms

The adverse effects observed in rodent hearts have not been studied in more detail, and 3,5-T2 concentrations in cardiac rodent tissue were found to be below detection limits (100) in a study aiming to determine THM concentrations in rat heart. Pinna et al. (29) determined 3,5-T2 concentrations based on immunoassay under careful methodological precautions in rat brains, and found quite high 3,5-T2 concentrations, which differed among brain regions but were low in hypothalamus and pituitary. In the study by Jonas et al. (39), who administered two doses of 3,5-T2, only the higher one led to antisteatotic effects in the liver while both suppressed the HPT axis. That effect of 3,5-T2 on hepatic metabolism, especially lipid metabolism, might be due to a remarkable accumulation of 3,5-T2 in the liver, which so far had not been reported in appropriate experimental paradigms. Whether 3,5-T2 accumulation is an issue of increased import or decreased export remains to be studied, as the classical transporters for thyroid hormones such as MCT8, MCT10, OATP, and LATs typically show low transport activity for 3,5-T2 compared to the other T4, T3, or 3,3′-T2, as the structural isomer of 3,5-T2 (101, 102). Considering these controversial and—with respect to species analyzed and models employed—conflictings observations, the initial enthusiasm of developing 3,5-T2 related agents as anti-steatotic drugs, devoid of thyromimetic activity and side effects, remains questionable, and more research is definitely needed if this path should be further taken. The available data sets also strongly discourage use of 3,5-T2, highly popular in the body-building and wellness scene and freely available via the internet, as a sliming or energy-boosting drug.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The Medical Ethics Committee of the relevant institutions approved each of the study protocols. The methods were performed in accordance with the approved guidelines. All included participants provided written informed consent in accordance with the Declaration of Helsinki. For the studies conducted at Georgetown University, the institutional review board approved the studies. The dialysis study was approved by the Institutional Review Boards of Hospital Divino Espírito Santo, Ponta Delgada, Açores-Portugal. The SHIP-TREND study was approved by the local ethics committee of the University of Greifswald and conformed to the principles of the declaration of Helsinki.

AUTHOR CONTRIBUTIONS

The manuscript has been drafted and written by JK. IL has performed the 3,5-T2 serum concentration measurements. KRe, ER, and KRi implemented the immune extraction studies with the 3,5-T2 monoclonal antibody. MP contributed the data and evaluation of the metabolome analysis. JA performed the dialysis study. MD and JJ planned, conducted, and evaluated the two clinical Jonklaas studies (53, 54). All authors have contributed parts of it, as well as read, edited, and seen the final submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2019.00787/full#supplementary-material

Supplmentary Data Sheet 1 | Serum concentrations of 3,5-T2 and 3-T1AM (relative to individual concentrations at t = 0 h) for the two kinetic studies administering Liothyronine (T3) to euthyroid volunteers (**Figure 5C**, upper panel) and hypothyroid patients (**Figure 5C**, lower panel).

Supplmentary Data Sheet 2 | Serum concentrations of 3,5-T2 and 3-T1AM for each individual receiving Liothyronine in the two kinetic studies presented in **Figure 6**. Upper panel **(A)**: 12 euthyroid volunteers with blood drawn over 72 h; lower panel **(B)**: 18 hypothyroid patients with blood drawn over 8 h.

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Hypothyroidism: Cardiovascular Endpoints of Thyroid Hormone Replacement

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Thyroid dysfunction, either thyrotoxicosis or hypothyroidism, represents an important cardiovascular risk factor. Heart disease is the leading cause of death for men and women in the United States. Cardiovascular disease is multifactorial and many efforts have been made to assess precipitants for optimal guideline-based, primary, and secondary prevention. Thyroid hormone receptors are present in the myocardium and endothelium, and small alterations in its levels could have significant effects in cardiac function. Specifically, overt hypothyroidism is associated with an increased risk for atherosclerotic cardiovascular disease due to metabolic and hemodynamic effects. Several concomitant factors like impaired lipid profile, low-grade chronic inflammatory state, increased oxidative stress and increased insulin resistance enforce this relationship. The last decade has seen a renewed interest on the impact of subclinical hypothyroidism on the cardiovascular system and whether or not it should be treated. The aim of this review is to provide current evidence of the effect of thyroid hormone replacement, either with levothyroxine mono-therapy or in combination with liothyronine, on specific cardiovascular parameters.

Keywords: hypothyroidism, L-T4, L-T3, heart failure, blood pressure, hypertension, carotid intima-media thickness, hyperlipidemia

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INTRODUCTION

Hypothyroidism presents in a wide biochemical and clinical spectrum from Subclinical (SH) to Overt Hypothyroidism (OH) and myxedema coma. Hypothyroidism is the most common endocrine disorder after diabetes and although standard treatment with Levothyroxine (L-T4) replacement is established as monotherapy, multiple symptoms remain unresolved despite maintaining a normal TSH. This led over the last two decades to several trials with combination treatment [Levothyroxine (L-T4)/Liothyronine (L-T3)] (1).

In a patient with intact thyroid gland, since symptoms of inadequate or lack of thyroid hormones are mostly non-specific, biochemical testing is the main diagnostic tool after excluding physiologic (like non-thyroidal syndrome) and/or testing interferences (like biotin effect). Typical signs and symptoms of hypothyroidism are correlated with the severity and duration of the disease, but are also associated with patient's age, sex, other comorbidities, and the etiology of hypothyroidism. Classic clinical findings in overt to severe hypothyroidism include: reduced deep tendon reflexes, skin changes, weight gain, reduced basic metabolic rate, cognitive dysfunction, and even hypothermia in advanced cases. Assessment of the signs and symptoms could be challenging especially if alterations in the thyroid hormone levels are subtle.

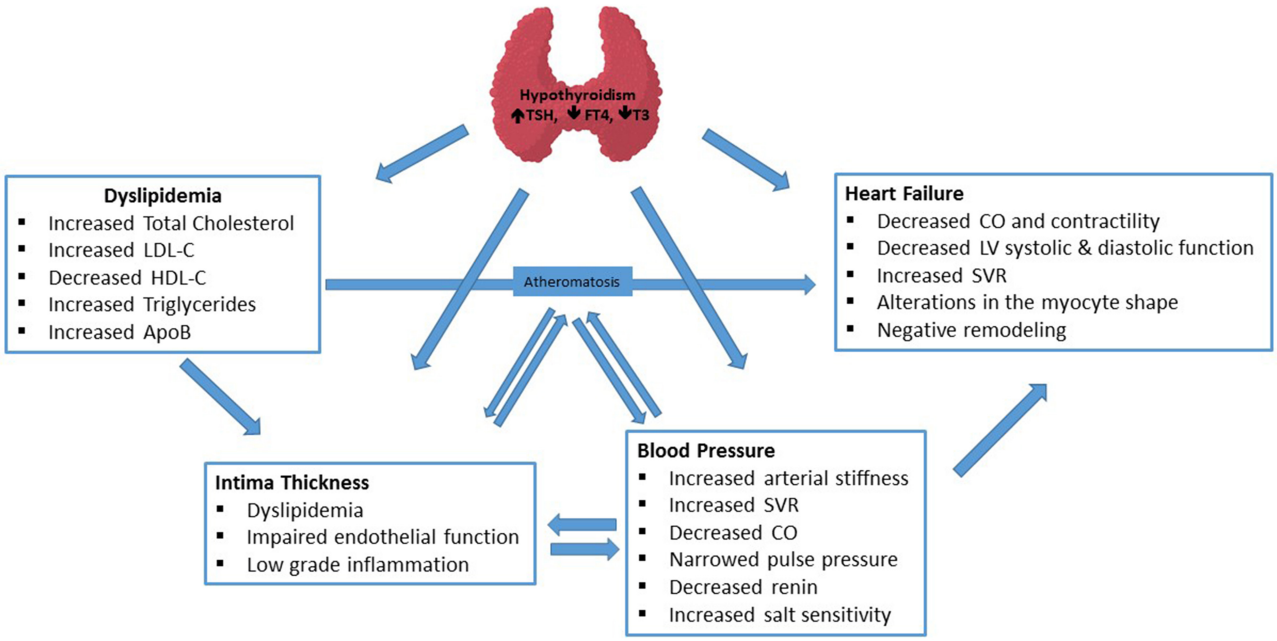

FIGURE 1 | Major mechanisms of the effects of Hypothyroidism on Cardiovascular parameters.

Thyroid dysfunction affects the cardiovascular (CV) system in all aspects from subclinical hypo/hyperthyroidism to overt hypo/hyperthyroidism. Several studies documented that OH and SH are associated with increased risk of coronary heart disease, atherosclerosis, and mortality (2) proportionally to the severity of thyroid failure, particularly among the patients with a TSH level $\geq$ 10 mIU/L.

Thyroid hormone has both genomic and non-genomic effects on CV system. While genomic actions are mediated by nuclear thyroid hormone receptors, non-genomic actions can be nuclear receptor-independent and occur at the plasma membrane level of cardiac myocytes and vasculature (3). In addition, several concomitant factors like impaired lipid profile, low-grade chronic inflammatory state, increased oxidative stress, increased insulin resistance also contribute to significant association of hypothyroidism with CV risk factors. Pathophysiologic mechanisms, briefly summarized in **Figure 1**, are very complex for each clinical endpoint and have been reviewed in detail elsewhere (4, 5).

The effect of thyroid hormone replacement in energy expenditure, body weight, and quality of life parameters will not be addressed in this article. In this brief review, we focused on the effect of thyroid hormone replacement on cardiovascular parameters: lipid panel, carotid intima-media thickness, blood pressure, and heart failure.

Abbreviations: SH, Subclinical Hypothyroidism; OH, Overt Hypothyroidism; L-T4, Levothyroxine; L-T3, Liothyronine; CV, Cardiovascular; C-IMT, Carotid Intima-Media Thickness; CAC, Coronary Artery Calcium; RCT, Randomized Controlled Trials; CKD, Chronic Kidney Disease; BP, Blood Pressure; HF, Heart Failure; LDL, Low Density Lipoprotein; IDL, Intermediate Density Lipoprotein; HDL, High Density Lipoprotein; VLDL, Very Low Density Lipoprotein; CETP, Cholesteryl-Ester Transfer Protein; PCSK9, Proprotein Convertase Subtilisin/Kexin Type 9.

METHODS

Literature search was performed in English through PUBMED, querying for key words: hypothyroidism, thyroid hormone treatment, L-T4, L-T3, heart failure, blood pressure, hypertension, carotid intima-media thickness, and hyperlipidemia. We focused on chosen endpoints and human studies.

EFFECT OF THYROID HORMONE REPLACEMENT ON LIPID PANEL

Hypothyroidism, both overt and subclinical, could affect lipid metabolism. This relationship has been well-described in literature since 1930 and it is implicated in the increased CV risk noted on these patients. It is reported that the prevalence of hypothyroidism in patients with hypercholesterolemia is 4.3% (6). In addition, current guidelines from the National Cholesterol Education Program, the American Association of Clinical Endocrinologists, and the American Thyroid Association recommend screening for hypothyroidism the patients with newly diagnosed hyperlipidemia prior to starting a lipid-lowering agent (6–9).

OH is associated with atherogenic lipid profile with elevated levels of total cholesterol due to elevated levels of Low Density Lipoprotein (LDL) and Intermediate Density Lipoprotein (IDL), hypertriglyceridemia, and increased Apo A1-and B1-lipoprotein

levels (10–12). There are multiple mechanisms involved in the pathophysiology of hyperlipidemia in hypothyroidism, such as: (a) Decreased number of LDL receptors in the liver resulting in decreased LDL uptake and accumulation (13), (b) reduced activity of the LDL receptor (14), (c) Increased LDL oxidation (15).

Hypertriglyceridemia is related to decreased activity of the lipoprotein lipase (LPL) (16), which results in decreased clearance of triglyceride rich lipoproteins and of the hepatic lipase (HL) (17), which hydrolyzes High Density Lipoprotein (HDL) and is involved in the conversion of IDLs to LDL and of LDL to small dense LDL (sdLDL). In another study, it was shown that low T3 levels are contributing to enhanced triglyceride hepatic synthesis (18).

The HDL-C levels are elevated in patients with hypothyroidism due to increased concentrations of HDL_2 particles, reduced activity of hepatic lipase and associated decrease in the catabolism of HDL_2s (19). In addition, reduced activity of CETP leads to reduced transfer of cholesteryl esters from HDL to Very Low Density Lipoprotein (VLDL), contributing to the higher levels of HDL-C (20).

Treatment with L-T4 significantly reduces the above documented effects in the circulating lipids. In OH, the therapy with L-T4 requires a period of 4–6 weeks to improve or correct dyslipidemia (21). Substitution treatment reduces the levels of LDL-C, total cholesterol, HDL and triglycerides, by thyroid hormone-mediated increase in the activities of lipoprotein lipase and hepatic triglyceride lipase (22). In some studies, it was shown that in patients who achieved euthyroidism with L-T4, the levels of lipoprotein A decreased (11, 12, 23). In other studies, there was no change in the levels of lipoprotein A before and after treatment with L-T4 (24, 25). Remnant lipoprotein concentrations, such as chylomicron remnants and very-low-density lipoprotein remnants, which are highly atherogenic, were lowered in patients who became euthyroid after treatment (22). For patients who received combination treatment with L-T4/L-T3, a statistically significant decrease in LDL-C and total cholesterol compared to L-T4 monotherapy was noticed after few months of treatment (26). In contrast, other trials did not show significant differences in the lipid panel of two-treatment groups (27–31).

Indeed, clinical guidelines recommend screening for hypothyroidism patients who present with dyslipidemia. It is also suggested that in patients with hypothyroidism and dyslipidemia, the administration of thyroid supplementation therapy to a statin, ezetimibe, or PCSK9 inhibitor might contribute to an enhanced and sustained effect (28, 29).

In SH, where the levels of TSH are elevated, but the levels of T4 and T3 are normal, a small percentage of patients are at risk to develop dyslipidemia, although this association is controversial, and the studies have been inconsistent. In a retrospective cohort study that aimed to determine the prevalence of thyroid function screening in patients with newly diagnosed hyperlipidemia, involving 8,795 patients, 49.5% of the patients had the thyroid function tests checked. From them, 11.1% had SH and 3.5% had TSH level of 5–10 mIU/L, suggesting that SH could be a secondary cause of hyperlipidemia and is associated with high risk of heart disease (9). Some of the studies have demonstrated that total cholesterol and LDL-C levels are elevated in patients with SH and decreased after the initiation of L-T4 (32–34). The effect of the levels of TSH to the levels of total cholesterol and LDL-C were stronger in the ages of 40–49 and 60–69 years old, compared to younger individuals, suggesting that SH might worsen the effects of aging on lipid profile (35). It was also shown that in patients with SH with higher serum TSH, there was a significantly increased serum Proprotein convertase subtilisin/kexin type 9 (PCSK9) levels which further contributes to a higher LDL-c level than the matched euthyroid participants (36, 37).

Serum HDL-C levels were found to be low (38, 39) or normal and there was no significant effect after L-T4 treatment (32, 33, 40). Serum triglyceride levels were elevated in patients with SH compared with euthyroid individuals, as it was evaluated in a meta-analysis that included 16 observational studies (41). On the contrary, other studies did not show change in triglyceride levels in SH, before and after treatment with L-T4 (10, 23, 32, 33, 40).

In SH, there is a statistically significant elevation of apolipoprotein B levels (42), which decrease after treatment with L-T4 (43). Similarly, apolipoprotein A levels were elevated in some patients with SH and decreased after the treatment with L-T4 (23). In other studies there was no significant changes in the apolipoprotein A levels and subsequent treatment with substitution therapy (32, 44). The levels of oxidized LDL-C were higher in patients with SH than in euthyroid controls and oxidative modifications of LDL-C may play a role in the initiation of atherosclerosis (45).

Currently no clinical trials reported the effectiveness of combination treatment (L-T4/L-T3) in patients with SH and dyslipidemia. Current evidence suggests that in patients with hyperlipidemia and SH, lifestyle modification, and lipid lowering medications should be started regardless of the decision for treatment with L-T4 (46).

CAROTID INTIMA MEDIA THICKNESS

Carotid intima-media thickness (C-IMT) is a non-invasive surrogate marker of subclinical atherosclerotic alterations and used to gauge the effect of interventions that decrease atherosclerosis. In European guidelines for prevention of CV disease, C-IMT of 0.9 mm is accepted as the threshold above which atherosclerosis progression occurs. The American Heart Association/American College of Cardiology Guidelines denote C-IMT and Coronary Artery Calcium (CAC) score as a class IIa recommendation for CV risk assessment in asymptomatic adults at intermediate risk for CV disease. An increase of 0.1 mm in the C-IMT was associated with a 10–15% increase in the risk of myocardial infarction and similarly with stroke risk (47, 48). In the case of hypothyroidism, the main etiology is postulated to be the endothelial dysfunction and arterial stiffness, in addition to other factors like dyslipidemia and inflammation (4).

Two separate meta-analyses investigated the effect of LT-4 therapy on C-IMT in patients with SH. First meta-analysis (49)

included nine trials (three RCT and six self-controlled study) and the second one (50) included the same nine trials plus two more. Both systematic analyses reported that LT-4 therapy reduced C-IMT significantly after long-term therapy (>6 months). The authors interpreted this effect as multifactorial, possibly due to improvement in total cholesterol, LDL-cholesterol, triglyceride levels; systolic and diastolic BP and flow-mediated dilatation with treatment. Subgroup analysis demonstrated the decrease in C-IMT was higher in subjects with baseline TSH > 10 mIU/l comparing with TSH ≤ 10 mIU/l.

A recent study compared the C-IMT in 40 OH and 30 SH female patients with euthyroid controls. In the group with OH, there was a highly significant increase in C-IMT in comparison with the control group (0.7 ± 0.2 vs. 0.45 ± 0.07 mm, $P < 0.001$), which was similar in the SH group (0.6 ± 0.2 vs. 0.45 ± 0.07 mm, $P < 0.001$). The authors also looked at the blood flow after heat-mediated vasodilation as a marker for endothelial dysfunction: comparing with euthyroid subjects there were significant impairments in both OH and SH group, more pronounced in the OH (51). Although these studies had small sample size, varied in duration and population characteristics, the signal in improvement in C-IMT was substantial and it may reflect another target in the armamentarium of modifiable CV risk factors.

A community-based study from China including 2,276 non-diabetic, euthyroid participants found a significant inverse relationship between serum free T3 levels and C-IMT (52) after excluding traditional risk factors for atherosclerosis. This is an interesting observation as most significant association was on the lower FT3 quartile, although it was still within the normal levels. Such association was also observed in a similar study that looked the association of free T4 levels and C-IMT in euthyroid subjects (53).

In contrast, another population-based cross-sectional study from Italy, involving 5,815 participants (age range 14–102 years old), did not show an association between subclinical thyroid dysfunction and increased C-IMT (54). SH group subjects were noted to have very mild thyroid dysfunction with an average TSH of 5.09 (4.41–6.84), which might have obscured subtle effects. Similarly, in an analysis of the TRUST trial, which included European population with mild SH, no significant difference in C-IMT with L-T4 treatment was found (55).

BLOOD PRESSURE

Hypertension (HTN) is a global health problem, affecting 26.4% of adult population (56) and is one of the modifiable risk factors in CV disease morbidity and mortality. Most of the cases involved have primary HTN, but ∼10% may have secondary causes, including endocrine ones. It is well-reported in literature that the incidence of HTN in cases of toxic goiter or myxedema is high and usually responds to treatment of the underlying thyroid condition (57). Specifically, hyperthyroidism is associated with systolic hypertension (58), while OH and SH with diastolic hypertension (59).

A large, cross-sectional population study of more than 30,000 patients showed a linear increase in BP with increase in TSH values even all were within the normal reference range. Comparing upper normal range of TSH (3.0–3.5) with the lower (0.5–0.99) the odds ratio for HTN was found 1.98 for men and 1.2 for women (60). Moreover, increased risk of pre-eclampsia has been reported in a study on pregnant women with SH in comparison to euthyroid women (61).

Diurnal changes occur in BP and under normal physiologic conditions a 10–20% reduction in BP occurs at night, which is called nocturnal dipping (62). Failure to show this pattern i.e., nocturnal non-dipping has been documented to be a sign of CV or metabolic complications. The loss of this nocturnal decline, i.e., the development of a non-dipping type of BP, is frequently observed in metabolic disorders and chronic kidney disease (CKD) and contributes to the development of CV disease. A recent trial reported reversal of loss of nocturnal dipping with LT-4 treatment in SH patients (63).

A meta-analysis investigating the effects of LT-4 treatment on BP in patients with SH included 29 studies (10 RCTs and 19 prospective follow-up studies) and concluded that LT-4 replacement therapy reduced the BP in the SH group significantly and may contribute to modifiable CV risk factors for these patients (64). On the other hand a large double-blind, randomized, placebo-controlled trial (TRUST) involving 737 elderly patients (65 year old or older) with SH showed no benefit from LT-4 therapy in their BP, however the BP reduction was not the primary endpoint in this study and the patient population was limited to 65 year and older individuals and cannot be generalized in all age groups or younger patients (65).

In trials with combination therapy (L-T4/L-T3), BP changes were not reported as a primary outcome measure, but as a secondary or other outcome. Two studies demonstrated no significant difference at baseline compared to combination treatment (29, 66). However, in one of them after 4 months treatment with either LT-4 or combination therapy, a reduction in diastolic BP was noticeable. Similar results were reported in another trial (in the T4 alone group) (67). Considering that these patients were not clinically and biochemically hypothyroid at the time of randomization, capturing the difference in small groups would be challenging and studies were not powered for this outcome.

In summary hypothyroidism in all spectrums, including overt and subclinical, may contribute to HTN and detailed evaluation of thyroid function is essential as part of the appropriate work-up.

HEART FAILURE

Despite major improvements in medical knowledge, application of technology and new medications, CV diseases, and particularly heart failure remains one of the major causes of morbidity and mortality in the developed world. Guidelines of American College of Cardiology/American Heart Association for the diagnosis and management for heart failure recommend investigating exacerbating conditions such as thyroid dysfunction, but without specifying the impact of different TSH levels or specific T3 levels.

Several studies showed that HF patients with OH or mild thyroid dysfunction had more hospitalizations and poor

prognosis, compared to patients who had normal thyroid function (68, 69). Another important observation was that about 15–30% of patients with HF reported to have low levels of T3, called "Low T3 syndrome" and this was also associated directly with the prognosis and severity of the HF (70–72).

The main findings reported in HF associated with low T3 syndrome can be summarized as: (a) Decrease in Left ventricle diastolic and systolic function, (b) Increase in systemic vascular resistance, (c) Decrease in cardiac contractility, (d) Renal function deterioration, and finally (e) cardiac output reduction. All these changes lead to alteration in cardiac myocytes and negative cardiac remodeling (5).

Reversible cardiomyopathy is documented in OH and myxedema cases and the pathophysiology includes genomic and non-genomic action of thyroid hormone in multiple levels at the myocytes and vascular system (46). The natural history of HF is characterized by progressive hypoxia, which in turn is a major driver for the ectopic expression of type-3 deiodinase, which is responsible for the shunting of T4 into reverse T3 (73). Additionally, the state of chronic inflammation associated with HF promotes the activity of type-2 deiodinase in the hypothalamus, inhibiting the release of TRH with a consequent decrease in TSH and overall thyroid hormone production. In the aggregate, these events generate a state of low T3 syndrome, which is commonly seen as an adaptive response. On the other hand, there is experimental evidence that T3 supplementation in animal models of HF has shown beneficial effects on myocardial function, suggesting that in advanced HF this condition is indeed maladaptive (4, 74).

Earlier studies showed improved cardiac output and exercise tolerance in non-ischemic HF patients with T4 replacement therapy, however they were small trials with short duration. T3 treatment in patients with low T3 levels was investigated in several studies. One of them included both patients with ischemic and non-ischemic cardiomyopathies, who were treated with T3 infusion. Their neuroendocrine profile significantly improved (heart rate, BNP, aldosterone and nor-adrenaline levels all decreased) and the ventricular performance increased (72). The study with thyromimetic DITPA (3,5-diiodothyropropionic acid), a thyroid analog treatment, did not pass phase II due to significant side effects of increased heart rate, gastrointestinal symptoms and no clear benefit in HF specific primary end points (75).

On the other hand, a European study tested T3 replacement for 3 months in chronic HF patients with low T3 levels and the primary end point of the study was left ventricular ejection fraction measured by MUGA-SPECT (76). This randomized placebo-controlled study did not support a beneficial effect of T3 treatment in this group of patients. However, one may argue that only 13 patients completed the study and their baseline ejection fraction (EF) was average 43%, so only mildly reduced. No side effects were noted in the treatment group.

Furthermore, all CV changes that occur during OH have been detected also in the SH cases, but to a lesser degree or different extent. Subtle changes in circulating thyroid hormone levels may have a significant impact on the CV system and subclinical thyroid dysfunction has been associated with a 20–80% increase in vascular morbidity and mortality risk (77–79).

In a pooled analysis of six prospective trials of HF, thyroid dysfunction was detected in more than 10% of patients, more being subclinical hypothyroid (8.1%). Both higher and lower TSH levels were associated with increased risk of HF event after adjustment of age, sex, and other risk factors (80). When a large cohort of pre-existing heart failure patients (Pen Heart Failure Study) evaluated for thyroid hormone status and CV composite endpoints, including ventricular assist device placement, heart failure, heart transplant or death, significant associations were noted: a three-fold increase in the risk of composite end point, if TSH $\geq$ 7and a two-fold increase, if there was isolated low T3 (81). In a study of 163 chronic HF patients with SH who were treated with L-T4 for 6 months, their physical performance was significantly improved, when normal TSH levels were reached (82).

Most recently, ThyroHeart-CHF trial is designed as a prospective, multi-center RCT to study the efficacy and safety of thyroid hormone supplementation in patients with chronic heart failure and SH. The study findings could have a significant impact on the discovery of new therapeutic targets and methods of HF (83).

CONCLUSION

Thyroid hormone interacts with and influences most metabolic pathways in virtually all organ systems throughout the entire life of the organism through genomic and non-genomic actions.

Hypothyroidism has a wide spectrum of clinical manifestations. Not only major changes, but also subtle alterations in the circulating pool of thyroid hormones may cause or contribute to CV risk.

Current data is limited by small RCTs, observational and small experimental studies. Developing well-designed, large, prospective longitudinal clinical trials to identify dose-effect relationship and specific populations that can benefit from L-T4, L-T3, or combination therapy should be in the agenda of thyroid and CV health researchers.

AUTHOR CONTRIBUTIONS

SY and AS have made a substantial, direct and intellectual contribution to the work, and approved it for publication. PB has helped during the early literature search.

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REVIEW
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Cardioprotection and Thyroid Hormones in the Clinical Setting of Heart Failure

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Ischemic heart disease is the main cause of morbidity and mortality worldwide and is becoming more widespread with population aging. Cardioprotection is a dynamic process characterized by mechanisms related to myocardial damage and activation of protective factors. Targeting these processes could be attractive as a new therapeutic strategy in the evolution of post-ischemic heart failure (HF). In this context, the role of thyroid hormone (TH)-mediated cardioprotection is supported by a number of findings regarding the modulation of neuroendocrine systems, inflammatory and oxidative stress status, pro-survival intracellular pathways, and epigenetic factors, its effects on cardiac angiogenesis, structure, and function and on the preservation of mitochondrial function and morphology, and its beneficial effects on cell growth and redifferentiation. Moreover, the numerous effects of TH on the heart involve genomic mechanisms, which include cardiac differentiation during the perinatal period and non-genomic action, directed toward the maintenance of cardiovascular homeostasis. This evidence suggests that there is an opportunity to treat HF patients with TH. This review is mainly focused on the clinical evidence of the role of the thyroid system in the complex setting of HF.

Keywords: cardioprotection, heart failure, epigenetic, thyroid hormones, subclinical thyroid disorders

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THYROID HORMONES IN THE SETTING OF HEART FAILURE

Heart failure (HF) represents a progressive chronic degenerative disease, starting with systolic or diastolic dysfunction of the left ventricle and evolving toward systemic disease, in which other organ dysfunctions and systemic responses are involved. The main cause of HF is ischemic coronary artery disease, with or without myocardial infarction, as a consequence of coronary blood flow reduction, myocardial necrosis, and the ischemic remodeling process. The clinical and social impact of HF is relevant considering its incidence, severity, and social costs (1–3) and also in association with progressive aging of the worldwide population (4–6).

Triiodothyronine (T3) and thyroxine (T4), produced by the thyroid gland, have multiple effects on the heart. Whether circulating T4 originates from the thyroid, T3 is produced peripherically by T4 5′-monodeiodination. Thyroid hormones (THs) influence both diastolic and systolic functions and have relevant effects on cardiac morphology and structure, coronary vasculature, cell metabolism, and cell protection, growth, and differentiation. As shown in **Figure 1**, the multiple effects of TH on the heart are mediated by different signaling pathways that have been clustered into genomic and non-genomic actions: THs regulate gene expression through specific nuclear α and βTH receptors (TRs) and regulate genes involved in metabolism, cell growth and differentiation. THs also exert non-genomic actions via interactions with cytoplasmic and

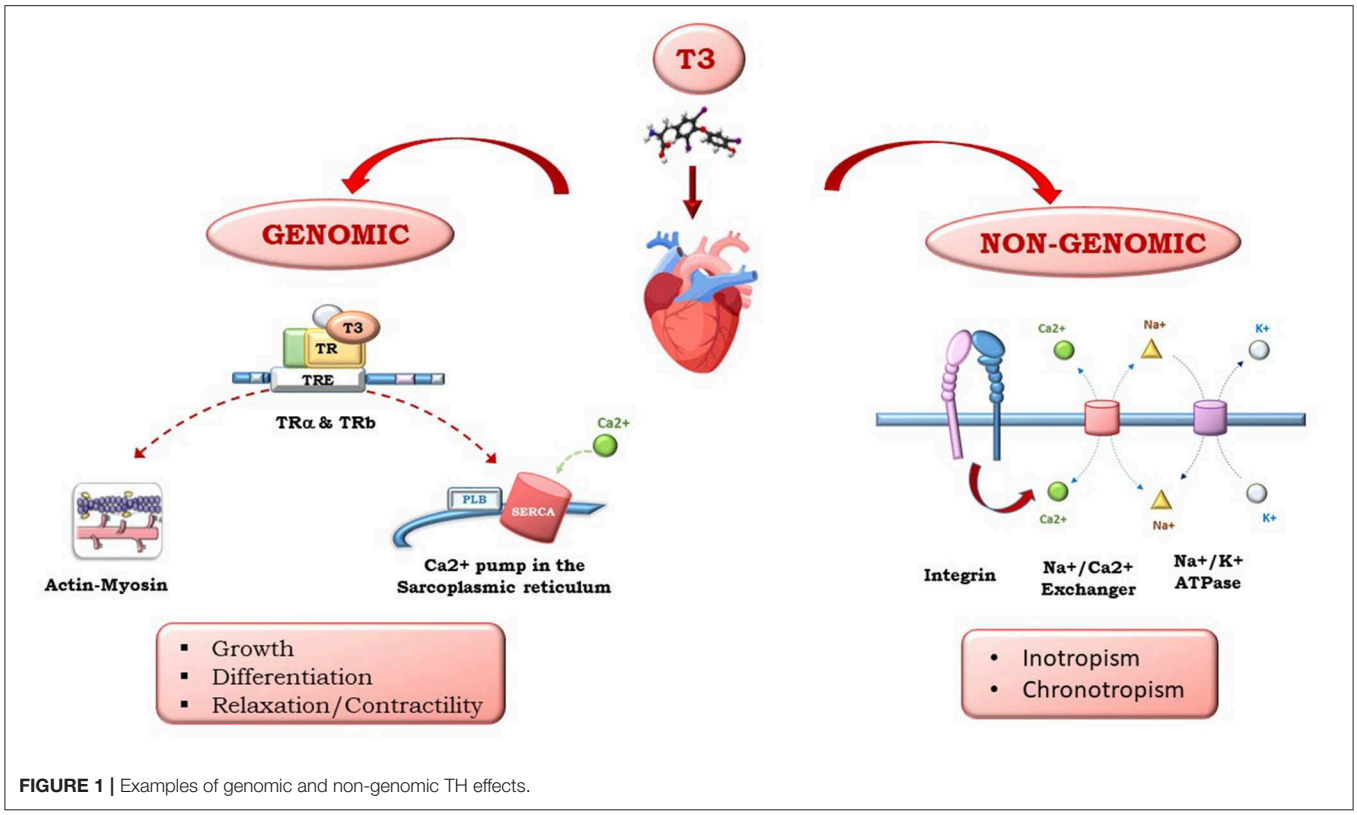

FIGURE 1 | Examples of genomic and non-genomic TH effects.

membrane-associated TRs, such as integrin αvβ3, that mediate TH action on the transport of ions across the plasma membrane and glucose and amino acid transport. THs act directly on the myocardial structure, regulating the interstitial collagen content within the myocardium, favor the development of coronary angiogenesis, thus increasing coronary flow reserve, and regulate cardiac function through chronotropic, inotropic, and dromotropic effects. Furthermore, THs also act through synergistic actions with inflammatory and neuroendocrine systems, as well as oxidative stress machinery, through direct action on the mitochondria. The high level of TH signaling integration with other systems is particularly relevant in HF. In fact, TH has an initial protective role, and its continuous activation results in toxic effects on all the systems implicated in HF pathophysiology.

THYROID AND HEART FAILURE IN THE CLINICAL SETTING: THE PROGNOSTIC IMPACT OF TH ABNORMALITIES

Hypothyroidism is the TH disorder that is most studied in experimental and clinical settings of HF. The studies are mainly focused on the prognostic role of hypothyroidism and on the effects of TH replacement therapy on cardiac function and clinics. The mild hypothyroid alterations investigated in HF clinical settings are subclinical hypothyroidism (SCH) and low triiodothyronine syndrome (LT3S). SCH is defined by 4.5 mIU/L ≤TSH≤ 20 mIU/L, with a normal T4 concentration. Actually, a TSH value of 10 mIU/L is the suggested cut-off for substitutive thyroid therapy. LT3S is defined as low T3 circulating plasma levels in the presence of normal thyroxine and TSH levels.

In the meta-analysis of Wang et al., LT3S incidence was higher in HF patients than in those with acute coronary syndrome, suggesting LT3S as a marker of chronic disease (7). In the context of acute decompensated HF, LT3S, and SCH are also considered markers of clinical severity, left ventricular dysfunction, and hemodynamic impairment (8–11). Accordingly, in the study of Rothberger et al., in which 137 patients with acute decompensated HF were enrolled, those with LT3S (48%) had a greater need for mechanical ventilation (12). Similarly, patients with SCH showed a more impaired clinical status. In the study of Sato et al., SCH was associated with lower oxygen consumption and higher mean pulmonary arterial pressure in comparison to euthyroidism (13).

In the clinical studies, there are conflicting results regarding the prognostic relevance of TH abnormalities, reflecting the heterogeneity of the population enrolled in terms of HF cause, sex, age, race, degree of TSH suppression, and duration of follow-up, modality of acquisition of the data and their fullness, time of TH sampling, and time of patient enrolment. Indeed, available studies include patients with both ischemic and non-ischemic HF and patients with stable and acutely decompensated HF. Furthermore, the events considered included cardiac and overall death, as well as hospitalization and arrhythmias. Finally, the chosen age range was variable (37–75 years). Moreover, in the

study of Okayama and Sato, patients with normal left ventricular function were also enrolled (14, 15). The study of Mahal et al., showing that in hospitalized HF patients with SCH there was no increase in mortality and major morbidity, was a retrospective population study in which the data were obtained from the Nationwide Inpatient Sample (NIS), using validated ICD-9CM (classification of diseases, 9th edition, clinical modification) codes for the identification of patients with HF and other comorbidities (16). Among 143.735 patients, half of them were above 80 years whereas a low proportion of patients were below 60 years of age (11%). This is a relevant factor if we consider that TSH values increase with aging, and this justifies the use of different reference intervals in old subjects, in particular in subjects 60 years old and older (17). Therefore, the high limit of TSH >4.0 mIU/l to define SCH can be restrictive in old people, and thus a larger TSH normal reference range should be used to avoid misdiagnosed SCH. This evidence should also partially explain the absence of clinical benefit from levothyroxine in older patients in terms of reducing cardiovascular events and mortality (18).

In the study of Frey et al., the absence of prognostic weight of SCH might be a consequence of the fact that TH replacement therapy was not an exclusion criterion of the study (8). Indeed, 27% of SCH patients were treated with levothyroxine. Usually, information on the persistence of the altered thyroid status, in the follow-up, may further increase prognostic stratification. In fact, when evaluated in the follow-up, the development of TH disorders was observed in patients with chronic stable HF without evidence of previous TH abnormalities (LT3S 12.5%, SCH 10.4%, overt hypothyroidism 6.2%), suggesting that it may be an important factor for HF progression (19).

It may be important to consider TH abnormalities in the context of a multiparametric approach in the prognostic stratification of HF patients, in which different clinical, biohumoral, and functional data are included. In this perspective, the study of Li et al. (20) showed that SCH was not an independent predictor of all-cause mortality after adjusting for other confounding factors, although patients with SCH had a higher incidence of overall mortality. On the contrary, the study of Wang et al. showed that FT3 was the stronger predictor of cardiac events, together with the extent of necrosis (21). In addition, cardiac death was significantly higher in patients with HF and LT3S in comparison with patients with a similar left ventricular ejection fraction but normal total T3. This finding indicates that LT3S is an independent predictor of mortality, adding prognostic information to conventional clinical and functional cardiac parameters, such as left ventricular ejection fraction (22). Moreover, the negative prognostic stratification is improved by combining measurement of brain natriuretic peptide (BNP; a hormone secreted by cardiomyocytes in response to stretching derived by increased ventricular blood volume, and a validated HF biomarker) with the presence of LT3S both in acute decompensated and in chronic compensated HF (23, 24). In another study by Sato, LT3S was associated with high cardiac and overall mortality, accompanied by high central venous pressure, lower nutritional status, and impaired exercise capacity (15). Moreover, the prognostic relevance of TH abnormalities may also be influenced by the fact that TH abnormalities occur in co-morbidities frequently observed in HF. In particular, in HF patients with hypothyroidism, renal insufficiency was significantly worse than in patients with normal thyroid function (25, 26). Also, in hemodialyzed patients, LT3S was strongly associated with cardiac death, and TH replacement therapy attenuated the rate of decline in renal function in chronic renal failure patients with SCH (27, 28). Therefore, although there are no relevant data on the potential association among TH abnormalities, HF co-morbidities, and HF, it is conceivable that these associations could result in a vicious cycle, leading to additive and detrimental effect on the clinical conditions and prognosis of HF patients.

TH TREATMENT IN HF PATIENTS

Several clinical studies report different data on TH replacement therapy regarding methodologies using short-term and long-term TH replacement therapy, T3 or T4 at different doses, and modality of administration. In addition, there are differences in the population cohorts in which these treatments are adopted, including HF patients with a normal or abnormal TH profile or with stable or unstable HF (29–37). Thus, conclusions are far from being definitive and widely shared by the scientific community.

Thyroxine Treatment

In stable chronic HF patients ($n = 10$) with idiopathic dilated cardiomyopathy, oral administration of T4 at a dosage of 100 μg/day for 1 week and 3 months (30, 31) was well-tolerated, inducing increased cardiac function and reducing systemic vascular resistance. Furthermore, the administration of dobutamine (10 μg/Kg/min) (a direct-acting inotropic agent and an adrenergic agonist that primarily stimulates the β1-adrenoceptors, increases myocardial contractility and stroke volume, also reducing peripheral vascular resistance and ventricular filling pressure) improved cardiac output and heart rate in T4-treated patients ($n = 10$), suggesting enhanced cardiac adrenergic sensitivity, in line with experimental data showing β1-adrenergic up-regulation via TH (38). Moreover, in the study of Curotto Grasiosi, T4 administration in HF-SCH patients ($n = 163$) improved functional capacity and physical performance (32). In acute decompensated HF patients ($n = 20$), unresponsive to conventional inotropic therapy and intra-aortic balloon counterpulsation, the positive hemodynamic effect of intravenous T4 administration (20 μg/h) was documented and maintained for enough time to complete surgical treatment consisting of heart transplantation or left ventricular device (33).

Triiodothyronine Treatment

In patients with HF, T3 administration has been applied both orally and intravenously (29, 34–37). In the study by Hamilton et al., patients ($n = 23$) with advanced HF (NYHA functional class III-IV) and low T3 levels and/or elevated plasma reverse T3 (rT3) values who received a high dose of T3 (T3 bolus of 0.7 μg/kg followed by 6-12 h T3 infusion to a total dose of 1 or 2 μg/kg) had a significant increase in cardiac output and a reduction

in systemic vascular resistance (34). In another two studies, constant infusion of L-T3 induced a progressive reduction in systemic vascular resistance and an increase in ejection fraction and cardiac output (29, 35). Moreover, in the same type of HF population ($n = 10$), a 3-day continuous T3 infusion induced an increased stroke volume and end-diastolic volume (29) without an increase in myocardial oxygen consumption. The increased end-diastolic volume can be considered to reflect the recruitment of residual ventricular filling reserves due to the effects of T3 on diastolic relaxation (39, 40). These results fit well with those observed after normalization of the thyroid state in patients with mild primitive hypothyroidism and without cardiac diseases. In these patients, left ventricular stroke volume, ejection fraction, and cardiac index significantly increased after synthetic TH replacement therapy, while blood pressure and heart rate did not change (41). Moreover, T3 infusion induced a reduction in the vasoconstrictor/sodium-retaining norepinephrine and aldosterone concentration, and in NT-proBNP (amino terminal fragment of brain natriuretic peptide precursor) levels, resulting in a circulating neuroendocrine biomarker profile improvement (29). By contrast, in 3 months of oral T3 therapy in HF patients ($n = 13$) with stable chronic HF and low T3 levels, a lack of benefits in cardiac function and in neurohormonal changes was observed (36).

The contrasting results observed can be attributed to the fact that, in the first study, patients had a lower ejection fraction and higher NT-proBNP, indicating a more severe clinical status (29, 36); furthermore, the type of administration and T3 dosage (intravenous vs. oral) were completely different, determining the different changes in TH levels (29, 36). In addition, the stability of circulating T3 levels was guaranteed with continuous intravenous infusion, whereas it is improbable that the same result would be gained with only two daily T3 doses, administered orally (29, 36). In a more recent study (37), in which T3 was administered long-term (6 weeks) at a dose of 0.025 mg/day per os in 39 patients, the left ventricular ejection fraction was improved, whereas NT-proBNP and inflammation markers were reduced and, interestingly, exercise capacity, evaluated by 6-min walking distance, was increased. Importantly, these contrasting results may suggest that the selection of HF patients and the modality of administration as well as the TH dose are determinants for a more effective therapy in HF. Moreover, these results underscore an important endpoint of TH treatment, which is to restore and maintain levels of circulating TH and TSH within their respective reference ranges.

Currently, it is not established whether T3 or T4 can be used in HF patients, which of them is more efficacious, and at which doses. Although T4 administration may represent the more physiological treatment approach, due to the fact that T3 is derived mainly from the T4 to T3 peripheral conversion, an increasing body of data suggest that the use of T3 is more effective. In fact, peripheral T4 to T3 conversion is impaired in chronic diseases such as HF, and thus the administration of T3 may be overcome by this step. This is a central aspect, since the cardiovascular system responds mainly to T3. Nonetheless, experimental findings have shown that only combined T3 and T4 treatment guarantees euthyroidism in all tissues of thyroidectomized rats, including myocardium (42).

Since the goal of TH replacement therapy in HF patients is to restore and maintain euthyroidism, supraphysiological doses ("pharmacological" hyperthyroidism) should be avoided. Until results from large, randomized clinical trials are available to confirm long-term safety and efficacy, the suggested substitutive dose of T3 should not exceed 0.2–0.4 μg/kg per day (that is, 15–30 μg per day, divided into two or three administrations) and about 1 μg/kg per day for L-T4 (that is, 50–100 μg once daily).

THYROID AND CARDIOPROTECTION

In general, the experimental studies showed the negative impact of altered thyroid metabolism on cardiac function, metabolism, cell protection, and surveillance, as well as on mitochondrial function and protection, and the reversibility of these alterations when restoring euthyroidism. This experimental evidence provides a strong indication of the potential role of thyroid hormones in cardioprotection.

The term cardioprotection includes all mechanisms contributing to heart protection by reducing or even preventing myocardial tissue injury and affecting multiple factors involved in cardioprotection (e.g., cytokines, cell growth, angiogenesis, and mitochondria). In this context, cardioprotection emerges as an attractive novel therapeutic strategy in the evolution of post-ischemic HF (43–45). There are several differences between acute and chronic HF myocardial damage. Acute ischemic HF myocardial damage is due to the abrupt occlusion of a coronary artery and reperfusion damage and ismore characterized by cellular death. In contrast, during chronic ischemic HF, a variety of remodeling processes and stimuli occur, which usually require up to several months (46), inducing changes in cardiomyocytes, extracellular matrix, and vasculature. The final result is the thinning of the infarct area and its expansion at the site of the necrotic border zone; hypertrophy and fibrosis of the remote zone likely occur as a direct response to increased wall stress (47).

Briefly, we report some experimental findings showing the multiple effects of TH on cardioprotection; these are illustrated in **Figure 2**.

TH and Pro-survival Intracellular Pathways

The cardioprotective effect of TH depends on the regulation of prosurvival pathways, including the activation and function of the phosphatidylinositol 3-kinase (PI3K)/Akt and PKC signaling cascade, the expression, phosphorylation, and translocation of heat shock protein 70 (HSP70) and HSP27, and the suppression of p38MAPK signaling (48). In particular, 3-day T3 treatment after acute myocardial infarction (AMI) had an antiapoptotic effect, reducing myocyte apoptosis in the border area, via Akt signaling (39). Pantos et al. (49) also reported the same outcome, with decreased p38 MAPK activation. Recently, TH was found to have a dose-dependent effect on Akt phosphorylation (50). Mild activation of Akt caused by a replacement dose of TH resulted in positive effects, while further induction of Akt signaling by higher doses of TH was accompanied by increased mortality and activation of ERK, a kinase linked to pathological remodeling

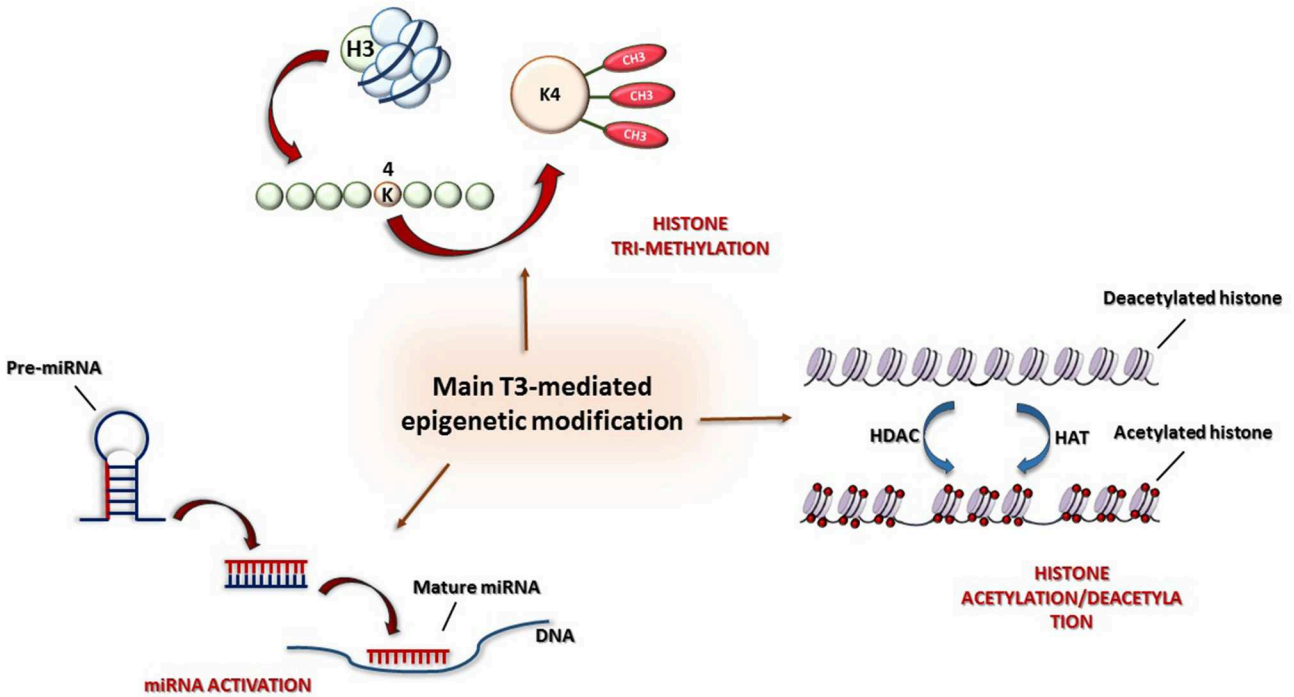

FIGURE 2 | Main epigenetic mechanisms: (1) Histone modification by acetylation/deacetylation, (2) tri-methylation of lysine 4 in histone H3 (H3K4me3), and (3) post-transcriptional control by miRNAs.

(51). This result suggests that the induction of pharmacological excess of thyroid function should be avoided, since it could have a negative impact on patients; therefore, the object of thyroid replacement therapy in HF patients should be the restoration and maintenance of euthyroidism.

TH and Myocardial Interstitium

THs also regulate collagen interstitial matrix biosynthesis, and their alteration can induce remodeling of the interstitial collagen content (52). In thyroidectomized rats, pro α1 (I) collagen and pro α2 collagen mRNAs and relative proteins increased significantly in myocardium, as did transforming growth factor (TGF)-β1. The activation of this molecular signaling induces a pro-fibrotic process through miRNA signaling (53). The activation of TGF-β1 signaling is hampered by early and short-term T3 supplementation, as documented in a rat model of ischemia/reperfusion, and this was associated with a reduction in scar size and preservation of cardiac performance (54).

TH and Coronary Vasculature

TH-induced cardioprotection is also exerted through the effect on coronary circulation. Hypothyroidism determines the reduction in arterial length and density, with the impairment of coronary vasodilation reserve, and this has been observed during hypothyroidism induced both by thyroidectomy and by propyl-thiouracil (55). The return to euthyroidism via T3 or T4 induced pro-angiogenic effects, with a restoration of arterial length and density (56). Similarly, in diabetic dysfunctioning heart, T3 treatment prevented the rarefaction of arteriolar resistance vessels with increased gene expression of TR-β, vascular growth factors (vascular endothelial growth factor A, VEGF-A), and endothelial nitric oxide synthase (eNOS) (57). TH-induced pro-angiogenesis is mediated by integrin αVβ3 that through the receptor site (S2) that binds both T3 and T4, activates extracellular regulated kinase (ERK) (58). Further, the expression of hypoxia-inducible factor 1 alpha (HIF-1a) through the interaction of TH with cytoplasmic TRβ and the activation of P13K signaling is another molecular circuit involved in T3 pro-angiogenesis (59).

TH and Mitochondria

TH is associated with the upregulation of mitochondrial proteins with functional relevance. In particular, TH mitochondrial protection is mediated by the regulation of tumor suppressor p53, whose network is activated under stress conditions such as AMI and HF and enhances the mitochondrial pathway of cell death (60). More recently, correction of post-ischemic LT3S has been shown to downregulate the mitochondrial-targeted noxious effect of protein p53, possibly through the upregulation of miR-30a (61). T3 treatment counteracts the decrease in miR30a level, limiting the activation of p53 and the cascade leading to mitochondrial injury and cell death in the border zone of myocardial infarction (62). Interestingly, in the human setting of post-ischemic HF, the levels of p53-responsive miRNAs (miR-192, miR-194, and miR-34a) were associated with adverse post-ischemic remodeling, indicating that they have the potential to be predictive of future ischemic HF (63).

TH-induced cardioprotection can also be provided by interaction between the thyroid system and the inflammatory, neuroendocrine, and oxidative stress systems, which, in turn, have a key role in the pathophysiology and clinical progression of HF. Another new area for consideration is the epigenetic mechanisms of TH regulation, which could open up new therapeutic opportunities.

TH and Inflammation

The so-called cytokine hypothesis highlights the importance of the dangerous effects of continuous activation of the inflammatory system, which favors the progression of heart damage and dysfunction, inducing apoptosis, mainly in myocytes and endothelial cells (64, 65). Experimental and human studies showed a cross-talk between TH and inflammation. In particular, Hajje et al. showed that propil-thiouracil-induced-hypothyroidism increased the plasma concentration of inflammatory markers C reactive protein (CRP) and cytokines (e.g., TNF-a and IL6) as well inflammatory gene markers (e.g., TGF-β1 and cTGF and Il1 and Mcp1). However, unexpectedly, the authors also observed a further increase in inflammatory markers despite improvement in cardiac function after the restoration of euthyroidism through T4 treatment, rendering these data difficult to interpret (66). Interestingly, in human cultured thyroid follicles, IL6 inhibits thyroid function, which might account for changes observed in LT3S (67, 68). Similarly, the administration of recombinant human IL-6 (rhIL-6) in animals resulted in an early decrease in serum T3 (69). The study of Lubrano et al. showed that, in patients with advanced HF, inflammatory markers TNFα, IL-6, and CRP correlated inversely with free T3, and this increase was significantly higher in patients with LT3S (free T3 $<$ 2 pg/ml) (70).

TH and Oxidative Stress

Elevation of oxidative stress (OxS; an imbalance between the generation of reactive radical species and antioxidant defense) has been demonstrated in every phase of HF development, and several biomarkers, such as those related to protein, lipid, or DNA peroxidation, have been associated with HF symptoms and disease (71). An excess in reactive oxygen species induces a reduction in cardiac function and myocardial growth, favors adverse remodeling with fibrosis proliferation, and thus participates in HF progression (72). TH is able to affect the antioxidant status, directly (e.g., iodide, I^-, can act as an electron donor and, as such, be effective as a scavenger of free radicals) or indirectly (e.g., stimulation or inhibition of the activity of antioxidant enzymes and free radical scavengers) (73). The effect of hypothyroidism on oxidative markers is a reduction of antioxidants and an increase in lipid peroxidation (74, 75). Conversely, the cardioprotective effects of TH have been associated with oxidative stress reduction. Specifically, the administration of T3 (2 μg/100 g/day) and T4 (8 μg/100 g/day) by gavage for 26 days in infarcted rats, which showed increased hydrogen peroxide and lipid peroxidation, decreased the reduced glutathione to oxidized glutathione ratio (GSH/GSSG), when compared to controls, reduced reactive oxygen species (ROS) levels, and improved cardiac function (76). Interestingly, in a recent AMI experimental rat model by Ortiz VD et al., the co-administration of carvedilol and TH reduced OxS (with an increase in GSH/GSSG ratio and an attenuation of the increase in NADPH oxidase activity and sulfhydryl group oxidation), and this effect was associated with the improvement of cardiac function. This study has a relevant clinical perspective, considering the potential additive effect of TH treatment over beta-blocker therapy, which is the state-of-the-art treatment of AMI patients (77).

TH and the Neuroendocrine System

It is well-known that neurohormonal activation represents a central determinant in the genesis and progression of HF. In this context, TH deficiency is frequently reported to reduce sensitivity and responsiveness to catecholamines due to decreases in β1 and β2 adrenoceptor density within the heart (78). In the study of Shao et al., rats with hypothyroidism induced by methimazole had a blunted response to isoproterenol, and this was associated with decreased β1 adrenoceptor (79). However, in the study of Brown et al., although the densities of β1 and β2 adrenoceptors increased in the hypothyroid state, there was an attenuated positive inotropic response to noradrenaline (80). In addition, in the cardiac relationship between TH and the sympathetic system, a potential role is also performed by β3 adrenoceptors, which, differently from β1 and β2, exert a negative inotropic effect (81). The study of Arioglu et al. showed that hypothyroidism induced by methimazole in rats was associated with an increase in the mRNA expression of β3 adrenoceptors and, contextually, the signaling pathway component of these receptors increased (82).

With regard to BNP, there is evidence that TH can modulate BNP release from both atrial and ventricular myocytes and that the plasma level of BNP decreases in hypothyroid rats (83). Moreover, in the study of Liang et al., after T3 treatment, BNP secretion increased 6-fold, BNP mRNA levels 3-fold, and BNP promoter activity 3–5-fold, leading to myocardial hypertrophy in neonatal rat ventricular myocytes (84). Furthermore, the study of Hajje et al. showed that hypothyroidism induced by propylthiouracil induced the expression of the fetal genes for atrial natriuretic peptide (ANP; hormone secreted in response to myocyte stretch in the cardiac atria, similar to BNP in its hemodynamic effects, with vasodilating properties) and BNP; this response was abolished by LT4 treatment (66). The potential effect of the reciprocal regulation of TH and the neuroendocrine system in HF is uncertain. As discussed below, continuous T3 infusion in HF patients induced deactivation of the neuroendocrine system, characterized by a reduction in plasma levels of noradrenaline, NTproBNP, and aldosterone. This neuroendocrine rearrangement may be mediated by a direct effect of TH on these endocrine systems and by an indirect effect due to the improvement in cardiac function (29).

A pathophysiological mechanism that could associate TH and neuroendocrine alterations in HF is that the continuous alterations of these systems may shift the action from adaptive to maladaptive responses. In this context, LT3S may initially be an adaptive process to minimize energy expenditure, but in its persistence can drive a maladaptive mechanism, becoming a factor in HF progression, in line with the negative clinical

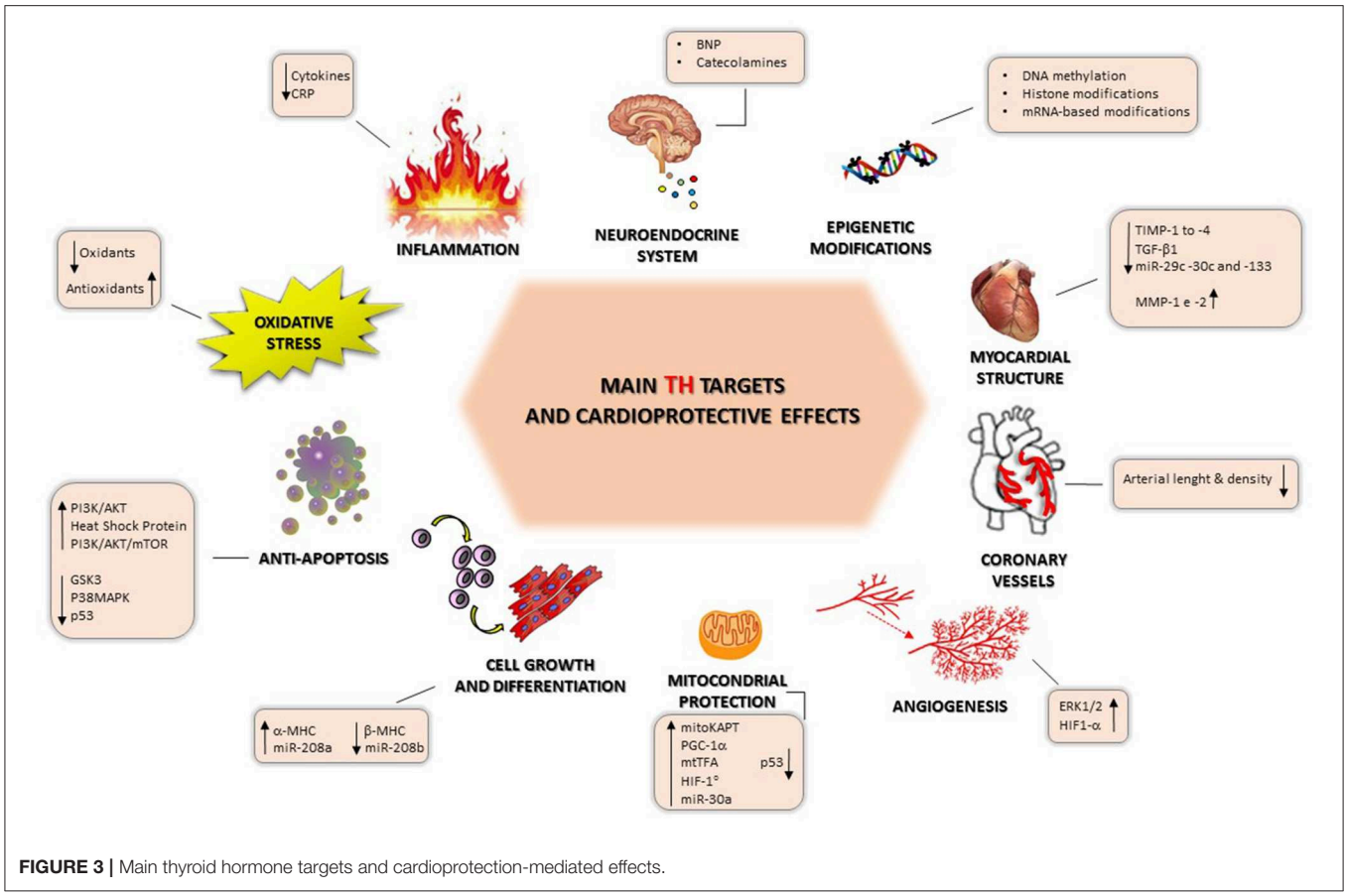

FIGURE 3 | Main thyroid hormone targets and cardioprotection-mediated effects.

and prognostic impact in the clinical setting and with the negative structural, histological, cellular, and functional effects experimentally documented in hypothyroid animal models.

TH and the Epigenetic Way: Chromatin Modifications

THs have a fundamental role in cardiovascular homeostasis, and alterations in TH signaling are associated with cardiac pathophysiology. More recently, it has become more and more evident that the regulatory effects of TH have to be investigated either at the genetic or epigenetic levels in order to individuate new therapeutic tools for the prevention and treatment of TH-dependent cardiac disorders.

Gene regulation occurs under the combined effects of transcription factors and cofactors and their interactions with promoter regulatory regions, and chromatin organization regulates the accessibility of these elements to the DNA.

Furthermore, besides genetic factors, cardiac gene expression is under the control of epigenetic modifications, which do not alter the underlying DNA sequence, either in normal or pathological conditions, being for this reason, reversible. Thus, epigenetic variations can give information beyond genotype, and because of their plastic patterns, they may be very suitable as key factors in the individuation of personalized therapies.

Previous studies have demonstrated the important influence of TH on the pathologic heart, especially on the cardiac MHC phenotype. The effects of TH on gene expression are complex and involve the interaction of several processes, including epigenetic events such as histone modifications and chromatin remodeling (85). Interestingly, transitions between different chromatin settings are the resultant of the balancing between factors that induce and maintain a silent state (corepressors) and elements favoring an active transcriptionally condition (coactivators). Alteration of this equilibrium results in a modification of the transcriptional state (86).

In particular, some studies have shown that the antithetical regulation of α and β-MHC genes by TH is under both genetic and epigenetic regulation (87). Both positive and negative regulation at the transcription level are driven by two opposite activities: histone acetylation and deacetylation, mediated, respectively, by histone acetyltransferase (HAT) and histone deacetylase (HDAC) (**Figure 3**). Furthermore, THs also influence histone methylation in chromatin; however, whereas histone acetylation always has an activating role on chromatin, histone methylation can have either a positive or a negative effect on transcription depending on the methylation site (88).

T3 exerts its effects through nuclear TRs, which bind specific TRE sequences in the promoters of target genes, mediating

positive or negative transcription states depending on the TREs involved.

The α-MHC gene is positively regulated by T3 whereas β-MHC is negatively regulated.

The molecular mechanism of T3 action has seen much study: in the absence of T3, TR is associated with nuclear corepressors (i.e., the NCoR/SMRT complex) (89), and, after deacetylation of lysine residues of histones H3 and H4 at TREs, chromatin undergoes structural alterations determining the repression of transcription. After T3 administration, corepressors detach from TRs and are replaced by a set of coactivators promoting histone acetylation (p160/steroid receptor and p300, TRAP/SMCC/Mediator complex) (90, 93). Furthermore, the TR-mediated activation is also associated with the tri-methylation of lysine 4 of histone H3 (H3K4me3) (87), which is considered, together with acetylation, the most conserved marker of gene activation (**Figure 3**). Interestingly, whereas histone methylation can be associated either with active or repressed genes, try-methylation of lysine 4 of histone H3 is only associated with active genes (86). Therefore, both histone H3 acetylation and H3K4me3 are post-translational indicators of gene expression. In particular, in T3-associated MHC modifications, H3K4me3 can be referred to α-MHC expression, whereas histone H3 acetylation to β-MHC expression.

TH and miRNA-Based Post-transcriptional Regulation in Cardioprotection

MicroRNAs (miRNAs) are small (20–24 nucleotides), single-stranded, non-coding RNAs that are involved in post-transcriptional modulation of gene expression (**Figure 3**). miRNAs belong to the non-coding RNA (nc-RNA) family, whose members have a functional role at transcriptional and posttranscriptional levels but are not translated into proteins. Several miRNAs have been individuated as crucial biomarkers of cardiovascular damage, mainly regulating gene expression throughout messenger RNA destabilization (54, 55). Even though the regulatory role of miRNAs in cardiac gene expression is well-documented, the association of miRNAs with regulatory mechanisms involving TH metabolism components in heart disease still has to be clarified (34). For example, it has been observed that, after myocardial infarction, type 3 iodothyronine deiodinase (D3, the main enzyme responsible for cardiac tissue hypothyroidism) and miR-214 are co-expressed in the heart. Since miR-214 is known to be activated in hypothyroidism, it has been hypothesized that it might play an important role in limiting the expression of D3 (35). Furthermore, D3 is considered to be one of the developmentally important genes activated in heart remodeling, associated with T3 reduction in myocardial infarction. Moreover, in pathological hypertrophy, TH directly modulates specific miRNAs, such as miR-208a, as activators, and such as miR-208b, as inhibitors (59). Isoforms of miR-208 are crucially involved in heart contraction and conduction, but pathogenic miR-208 overactivity possesses pro-hypertrophic, pro-fibrotic, and arrhythmogenic properties (60). The administration of specific single-stranded oligonucleotides, termed antagomir, can inactivate pathological miRNAs (61). In particular, targeted silencing of miR-208a, directly involved in the regulation of cardiac stress response, reduces cardiac remodeling and improves cardiac function during heart failure (59, 62).

Recently, the role of miR-27a as a modulator of thyroid hormone signaling has been demonstrated in the β-MHC gene, via TRβ1, in both *in vivo* and *in vitro* studies. In mouse hearts treated with transverse aortic constriction, which results in after-load cardiac hypertrophy, β-MHC gene expression increased whereas TRβ1 was downregulated. Contextually, miR-27a was upregulated, suggesting its association with the developing cardiac hypertrophy in terms of β-MHC regulation via TRβ1 (91).

CONCLUSION

The TH system plays a multilevel role in cardioprotection after HF due to the effects on molecular pathways and cardiac function and structure, as confirmed by the large amount of available experimental results. In the clinical setting, the majority of data show altered TH metabolism to have a negative prognostic effect. In view of these findings, the question of whether it is beneficial to treat patients with HF and abnormal TH patterns is not yet completely clear, although very preliminary clinical evidence showed potential positive effects. In this respect, it should be said that the recently updated guidelines of the American Association of Clinical Endocrinologists, in conjunction with the American Thyroid Association, introduced advice for which TH treatment could be considered in patients with HF and TSH levels exceeding the high normality reference limit up to 10 μIU/mL (92). The advice concerns the restoration and maintenance of a normal TH status in HF patients, avoiding over-treatment, which implies the dangerous consequences of pharmacological-induced hyperthyroidism. Large multicenter trials documenting the efficacy and safety of TH treatment in HF are still lacking. Future clinical studies are expected to evaluate which type of hormone (T3 or T4) is more indicated to be administered and by which route, at which dose, and with what timing in order to offer the safest and most effective TH treatment in HF patients. Other possibilities, such as those explored in the inflammatory or oxidative stress scenario (e.g., downregulation of GRK2 expression, use of antioxidants such as N-acetylcysteine, selenium, or vitamin D), may represent alternative/additive therapeutic tools to TH administration, although this research area is still in its infancy. In particular, whether biomarkers belonging to other pathways involved in the thyroid-HF axis (e.g., epigenetic modification, cell growth, and differentiation, myocardial hypertrophy, apoptosis, mitochondrial functioning, neoangiogenesis, and fibrosis) may serve as targets of a replacing or supplementary strategy to TH treatment remains an interesting field for future research.

AUTHOR CONTRIBUTIONS

AP: concept definition and writing and final review. FM and CV: writing and review. LS: writing, review, and figures.

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Design of the Optimal Trial of Combination Therapy

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Patients who take levothyroxine monotherapy to treat hypothyroidism frequently experience residual symptoms despite TSH testing at target levels. Trials have been conducted to evaluate the potential benefit of combination therapy with levothyroxine and liothyronine, though results have not consistently demonstrated benefit. In addition to randomization, placebo-control, and masking, four additional design choices to consider include the study population, dosing strategy for levothyroxine and liothyronine, primary and secondary outcome selection, and statistical power. A thoughtful design that considers these features will increase the likelihood that a combination trial will be considered definitive and finally resolve the important question of whether combination therapy with levothyroxine and liothyronine is a better thyroid replacement strategy than levothyroxine monotherapy.

Keywords: levothyroxine, LT4, liothyronine, LT3, hypothyroidism, thyroid hormone replacement, clinical trial

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INTRODUCTION

Clinical trials are difficult to design and execute, requiring multiple choices that affect feasibility of trial conduct and generalizability of the results. The best clinical trials are valid, due to good design and execution, and novel, answering new questions that are clinically important. The ultimate goal of a clinical trial should be to change clinical practice.

Key features of clinical trial design include randomization, which minimizes confounding, and masking, which minimizes bias. Randomized controlled trials are the only study design to address causality, and they provide Level 1 evidence (1). The shortcomings of clinical trials are that they are only able to address a specific question in a specific population, difficult to execute, and expensive.

The thyroid produces two major hormones, thyroxine (T4), and triiodothyronine (T3), in a 14:1 ratio. T4 has a longer half-life than T3 does, and the majority of circulating T3 is subsequently produced by peripheral deiodination of T4. A sodium salt of T4 that can be orally absorbed—levothyroxine (LT4)–was first introduced into the US market in 1949 for treatment of hypothyroidism. LT4 remains the recommended medication for thyroid hormone replacement today (2).

However, 35% of patients who are receiving recommended treatment with LT4 have impairment in psychological well-being (3). In addition, patients who are taking LT4 therapy have higher free T4 and lower T3 levels than euthyroid counterparts who are not taking LT4 (4). It has been suggested that LT4 therapy is not complete replacement therapy, and that therapy with a combination of LT4 and liothyronine (LT3) would better replicate normal thyroid physiology and therefore reduce hypothyroid symptoms. Although the clinical trials that have been performed to assess whether combination therapy with LT4 and LT3 is superior to LT4 alone have not demonstrated clear, consistent evidence to support combination therapy (2), this question remains unanswered in the eyes of many patients and clinicians, who point to design weaknesses in these trials.

The best clinical trials randomize treatment assignments, have a placebo or other control, and mask participants, investigators, and anyone assessing outcomes to the treatment assignment. Design of the "optimal" trial of combination therapy requires particular attention to four additional design features: the study population, dosing strategy for LT4 and LT3, primary and secondary outcome selection, and statistical power.

STUDY POPULATION

The selection of the study population of a clinical trial affects both the size of the trial and its generalizability. A larger impact is generally seen from interventions in patients with more severe disease, thereby reducing the number of patients needed to enroll in the trial. However, any selection criterion will impact the generalizability of results to patients who were excluded based on that criterion.

Four major decisions about inclusion and exclusion criteria affect the design of a trial of combination therapy. The first is whether patients should be restricted based on the LT4 dose at study entry. Patients who are taking a dose of LT4 that is below a full replacement dose (widely considered to be 1.6 mcg/kg/day) are likely to have residual thyroidal production of T4 and T3 and may not derive as much benefit from the addition of T3 as those who have no endogenous thyroid function. In addition, depending on whether a fixed dose or a fixed ratio of T3 is used, variability in the baseline LT4 dose may affect the feasibility of achieving similar LT4:LT3 ratios across the study population.

Second, whether enrollment should be limited to symptomatic patients or expanded to any LT4 user is an equally important design decision. This decision is largely impacted by whether the primary outcome is based on patient-reported or physiologic outcomes. Enrolling only patients who have residual symptoms despite taking LT4 doses considered adequate based on thyroid function testing increases the likelihood of detecting symptomatic benefit from combination therapy. Patients who feel good at baseline are unlikely to feel better with an alternative therapeutic regimen. However, if the primary outcome involves testing physiologic changes, this restriction would not be required.

A third consideration is the etiology of hypothyroidism: autoimmune or due to destruction or removal of the thyroid gland. Patients with an autoimmune etiology of hypothyroidism are more likely to have other autoimmune syndromes in addition, leading to a greater likelihood of symptoms attributable to non-thyroid conditions. Patients who had surgical removal of their thyroid due to structural disease do not have underlying autoimmune thyroid conditions and are therefore a more homogenous population. However, the group with hypothyroidism due to autoimmunity represents a larger section of the community of patients with hypothyroidism, impacting generalizability if this group were excluded.

The fourth decision is whether to incorporate low baseline T3 levels as an inclusion criterion. Obtaining an accurate measurement of serum T3 is challenging due to assay variability and biological variability in the setting of caloric restriction. An additional challenge to using T3 levels as an inclusion/exclusion criterion is the lack of knowledge about what level represents true T3 deficiency. The current T3 reference range lower limit represents the bottom 2.5% of the distribution of T3 levels in a population of individuals without thyroid disease. It is possible that patients above this range could still experience problems related to T3 deficiency. One caveat is that it seems unlikely that symptomatic patients with T3 levels in the upper half of the distribution of T3 levels are experiencing issues attributable to T3 deficiency.

DOSING STRATEGY OF LT4 AND LT3: THE INTERVENTION

This will likely be the most critical design feature for the uptake of trial findings by endocrinologists. There are four possible dosing goals: fixed dosing of a physiologic ratio of LT4:LT3 without titration based on thyroid function testing, variable dosing to achieve a TSH level within a specific window (e.g., 0.5–2.5 mU/L), variable dosing to achieve free T4 and T3 levels within reference ranges (or a narrower window within these ranges) irrespective of the TSH level, or a combination approach titrating to both serum TSH and T3/T4 ratio. Each approach has its advantages and disadvantages. However, abandonment of TSH testing as a guiding parameter for dose strategy would be counter to years of precedent in which TSH has been accepted as the primary thyroid function test. Furthermore, if the combination therapy and control groups differ with respect to their TSH concentrations at study conclusion, critics will argue that any differences in outcome were due to either underdosing or overdosing in one of the groups, threatening study validity.

Since LT4 pills are color coded by dose, masked titration of LT4 will require either manufacture of multiple doses of LT4 without dye or overencapsulation of commercially available LT4 with difficult to break capsules. The 5 mcg dose of LT3 is the only available dose and an identical placebo can easily be manufactured. If additional dose formulations of LT3 or a sustained release LT3 preparation were to become available, a more physiologically refined combination LT4/LT3 intervention could be tested. Neither is available at this time.

If consistent levels of T3 are desired, either twice or three times daily dosing of LT3 is required (5), with twice daily dosing preferred for patient convenience. LT4 and LT3 differ in their absorption, with higher variability in LT4 than in LT3 absorption. This is a drawback to using a fixed ratio of LT4/LT3 without titration to any thyroid function tests.

PRIMARY AND SECONDARY OUTCOMES

The primary outcome should be the efficacy outcome that will most influence clinical practice. At the completion of the trial, if the analysis shows no statistically significant difference between groups in the primary outcome, regardless of the secondary outcomes, the overall interpretation of the trial will be that combination therapy is not different from standard of care therapy.

Which efficacy outcomes will change clinical practice? The primary reason that patients pursue combination therapy is to ameliorate symptoms of hypothyroidism that can be found in a simple internet search (6). Physicians also want their patients to feel better. For both of these groups, the change in thyroid symptom scores would be the best primary outcome. In addition, endocrinologists want to understand the physiologic effects of different regimens across the multiple physiologic systems affected by thyroid hormones, including metabolic, cardiovascular, cognitive and musculoskeletal outcomes. The best clinical trials tend to have "hard" clinical outcomes that represent discrete, clinically relevant events. There is no single primary outcome that will satisfy all of these conditions.

The primary outcome that will affect the broadest group of individuals is one that assesses thyroid-related symptoms. Multiple questionnaires exist, though in a systematic review of quality of thyroid-specific health-related quality-of-life instruments, the Thyroid-Related Quality of Life Patient-Reported Outcome (ThyPRO) questionnaire was recommended for patients with hypothyroidism (7). Validated versions include the full 85-item ThyPRO (8–12) and the 39-item ThyPRO-39 scale with the 22-item ThyPRO Composite QOL scale (13). The ThyPRO-39 Composite QOL scale is based on 22 items from the Tiredness, Cognition, Anxiety, Depressivity, Emotional Susceptibility, Impaired Social Life, Impaired Daily Life and Overall QOL scales of ThyPRO. Item responses are scored 0 for "Not at all," 1 for "A little," 2 for "Some," 3 for "Quite a bit," and 4 for "Very much"/"Completely."

Questionnaires may not adequately capture the elements that underlie patient preference. All studies should include assessment of patient preference for the randomized regimen over the LT4 regimen prior to randomization, as well as whether the patient believed they were randomized to combination or standard LT4 therapy. Maintenance of masking is critical for ensuring that the assessment of patient preference is not biased.

Secondary, physiologic outcomes should be selected based on the following properties: responsivity to changes in T3, participant burden, availability at multiple centers, standardization across centers, frequency of measurement, and cost. Across each domain of physiologic outcomes, there is a range of testing available for use in the clinical research setting. Possible tests of metabolic efficacy include objective measurements of weight and waist circumference, resting energy expenditure measured with a metabolic cart, and use of actigraphy for activity monitoring. For cardiovascular efficacy, tests include a lipid profile, resting pulse rate, blood pressure, echocardiogram, brachial artery flow mediated vasodilation, VO2 max testing, and measurement of carotid intimal medial thickness. Assessments of cognition should include tests of executive function and memory that are easy to administer. An example is the fluid cognition composite score of the NIH Toolbox cognition battery (14). Potential musculoskeletal efficacy outcomes include bone biomarkers (e.g., C-telopeptide), DXA scans for bone density and body composition, hand grip strength, and tests of physical function, such as the Short Physical Performance Battery (SPPB) or the 400 meter walk test.

Safety assessments are also necessary in any trial of combination therapy. The same issues of anticipated relationship to LT3 use, participant burden, and cost to measure are as relevant for safety outcomes as for efficacy outcomes. Changes in symptoms of thyrotoxicosis should be specifically assessed in addition to a general assessment of adverse events. The primary concerns with LT3 are due to the chronotropic effects of T3 and include sinus tachycardia and atrial arrhythmias. The incidence of atrial fibrillation should be collected through self-report and EKG assessments at visits. Wearable 2-week cardiac monitors are now available at a reasonable cost that have the advantages of smaller size and better portability than Holter monitors.

STATISTICAL POWER

The primary outcome of a study has to be frequent enough and the variation small enough to demonstrate a statistically significant and clinically significant difference. Enriching the study population with a higher risk group, for example, those taking higher replacement doses of LT4, allows for a smaller sample size. The primary and secondary outcomes must be prespecified in the trial protocol and a clinical trials registry prior to study enrollment. The trial protocol should also include prespecified subgroup analyses, such as by etiology of hypothyroidism, baseline T3 level, or genetic background (type 2 deiodinase or MCT8 single nucleotide polymorphisms). It should also include techniques for management of statistical significance in the face of comparisons across multiple outcomes, possibly including hierarchical analysis of secondary outcomes.

One possible outcome of a combination therapy trial is that there is no statistically significant difference between combination therapy and standard LT4 therapy. This will raise questions about acceptance of the results of a null trial. Any combination therapy trial should be adequately powered to detect the minimal clinically important difference (MCID), not just a statistically significant difference. The MCID can be difficult to determine and usually requires confirmation via validation studies. It is important that the primary outcome has a widely accepted MCID to ensure that null results are accepted as readily as positive findings.

DISCUSSION

Recommendations for Design of the "Optimal" Trial

Patients taking LT4 doses close to the anticipated full replacement dose, at least above 1.2 mcg/kg/day, that is at least 100 mcg/day should be enrolled, both for feasibility of combination dosing at a physiologic T4:T3 ratio and for increased likelihood of benefit in those with less endogenous thyroid function. They should also have symptoms that are potentially attributable to hypothyroidism, since symptomatic improvement will be the primary driver of the clinical impact of combination therapy. Identification of individuals, based on symptoms or findings, who may actually benefit from LT3 is critical. Enrollment of patients with low or no symptom burden is an area of criticism of previous

trials of combination therapy. TSH concentrations should be within the reference range at baseline, demonstrating appropriate control on LT4 therapy prior to enrollment. Including both patients with autoimmune thyroid disease and those with hypothyroidism due to removal or destruction of the thyroid would be ideal, with stratification of the analysis by group. Study budget would be a key consideration for this strategy. Baseline T3 levels are not likely to be helpful for determining study inclusion, though they should be evaluated in a subgroup analysis.

Physiologic dosing of T3 should be used in twice daily doses, for example, replacing 25 mcg of LT4 with 5 mcg of LT3 twice daily. Although this will result in a different ratio of LT4 to LT3 dosing in an individual whose baseline LT4 dose is 100 mcg vs. 200 mcg, with the currently available LT3 doses and the logistics of placebo control, this is the most feasible option for starting dose. Maintenance of masking, either through generation of identical placebos or overencapsulation, is important for maintaining the study integrity. At the end of the study, while still masked, participants should be asked to guess the therapy to which they were assigned and their preference compared with their LT4 regimen prior to study initiation.

The primary outcome should be a symptoms assessment, such as one of the ThyPRO questionnaires, and a limited battery of secondary efficacy outcomes should be performed, such as weight, lipid panel, resting heart rate, a cognitive battery, and bone biomarkers. These would require limited equipment and training to execute. Additional measures to consider are resting energy expenditure and a DXA scan for bone and body composition. Assessments of safety should include hyperthyroid symptoms, tachyarrhythmias, and adverse events. All assessments should be performed at baseline, shortly after randomization, and repeated over the course of the trial to assess early and sustained responses. The duration of the trial should be at least 1 year in order to assess persistence of efficacy and safety over sufficient duration. A parallel design is preferred due the long study duration and concerns about carryover effects and the impact of dropouts in a crossover design. Subgroup analyses, such as by genetic polymorphisms, should be prespecified.

Additional Trial Considerations

The optimal clinical trial of combination therapy will require substantial funds and a significant commitment from study investigators and participants to implement. There will likely only be one large trial funded to answer this question. Design choices affect the validity and generalizability of a trial, which in turn affect the interpretation and clinical impact of the results. A thoughtful design that considers the features outlined above will increase the likelihood that a combination trial will be considered definitive and finally resolve the important question of whether combination therapy with LT4 and LT3 is a better thyroid replacement strategy than our current standard of care of LT4 monotherapy. If the clinical trial fails to show superiority of one of the regimens, then additional considerations, such as ease of adherence and cost should be incorporated into therapeutic recommendations.

AUTHOR CONTRIBUTIONS

AC drafted and edited the manuscript.

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Combination Therapy for Hypothyroidism: Rationale, Therapeutic Goals, and Design

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Hypothyroidism is a common condition with a wide spectrum of etiologies and clinical manifestations. While the majority of patients affected by hypothyroidism respond well to levothyroxine, some patients do not and complain of symptoms despite adequate replacement. There is evidence in experimental models of hypothyroidism that levothyroxine alone may not be able to deliver an adequate amount of T3 to all the tissues targeted by the hormonal action, while liothyronine/levothyroxine combination therapy can. The results of clinical studies directed to assess the effectiveness of liothyronine/levothyroxine combination therapy on the amelioration of hypothyroid symptoms have been disappointing. Most of the trials have been short and underpowered, with several shortcomings in the study design. There is consensus that an adequately powered clinical trial should be developed to prove or disprove the efficacy and effectiveness of therapies other than LT4 alone for the treatment of hypothyroidism, and to assess which group of patients would benefit from them. Here we present some considerations on the technical aspects and necessary tradeoffs in designing such a study with a particular focus on study population selection, choice of endpoints, and study drugs formulation and regimen.

Keywords: hypothyroidism, liothyronine, levothyroxine, combination therapy, pharmacokinetics

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INTRODUCTION

The majority of the patients affected by hypothyroidism are successfully treated with levothyroxine (LT4), which is considered by U.S. professional organizations to be the standard of care for this condition (1, 2). Unfortunately, a sizable minority of patients, up to 40%, still complains of hypothyroid symptoms despite achieving the target TSH (3, 4), resulting in repeated dose adjustments, additional testing, patient dissatisfaction, and provider frustration. Obvious advantages of LT4 thyroid hormone replacement therapy include well-known pharmacokinetics characteristics which allow for daily administration, its safety profile, and the wide array of dose strengths which allow for precise titration. Moreover, the use of TSH as proxy of euthyroidism provides a reliable therapeutic target for dose adjustments. LT4 therapy is based on the assumption that the peripheral conversion of exogenous T4 is able to provide adequate supply of T3 to the various targets of the hormonal action, and that a state of pituitary euthyroidism (as indicated by a TSH within the normal range) equates to a state of generalized euthyroidism. Of interest, while LT4 therapy is extremely effective in normalizing TSH, it does not appear to completely normalize the serum concentrations of T4 and T3, since the ratio T4:T3 is skewed toward an increase of T4, while T3 levels are normal to low (5). The clinical significance of these changes in thyroid hormone is not clear, but it may reflect a state of relative imbalance in the thyroid hormone axis homeostasis, which in turn could cause the persistence of hypothyroid symptoms in some individuals.

The addition of liothyronine (synthetic T3, LT3) to LT4 in different LT3:LT4 combination therapy schemes, or the use of desiccated thyroid extracts (DTE), are attempts to replicate the endogenous production of thyroid hormone. The scientific rationale for these treatments is based on the landmark experiments of Morreale d'Escobar in an elegant experimental model of hypothyroidism, which demonstrated that LT4 alone is not sufficient to reverse hypothyroidism in all tissues (6), and only administration of a combination of LT3 and LT4 could restore normal tissue content of T3 (7). Contrary to rodents, in humans the majority of the thyroid gland production is T4, which is then converted to T3 in the peripheral tissues. By mathematical modeling, Pilo and colleagues have estimated that the daily release of T3 from the thyroid gland is approximately 3.3 mcg * m^{-2}, while the peripheral conversion of T4 account for another 12.7 mcg * m^{-2} (8). These data have provided the theoretical underpinnings to design therapeutic regimens aimed to restore the "physiologic" concentrations of thyroid hormone.

Several clinical trials (**Table 1**) have been conducted with various treatment schemes, duration, and endpoints (9–16, 18–24). The results of these studies have been inconsistent, and in the aggregate LT3:LT4 combination therapy or DTE (25) have not shown superiority to LT4 in relieving symptoms of hypothyroidism. Nonetheless, some patients treated with combination therapy or DTE showed remarkable improvement in some of their symptoms, raising the question of how to identify which subgroup of patients could benefit from such treatment.

The need and design of additional trials aimed to assess the efficacy and effectiveness of LT3:LT4 combination therapies or DTE in the treatment of patients with hypothyroidism is being actively debated among researchers and practitioners. On the one hand, the inconsistency of the data obtained in the previous trials tends to dissuade from embarking on what would be another negative study; on the other hand, one could argue that a rigorous study design based on rational decisions on study population, outcomes, and drug formulation could be able to address an unresolved prevalent and disabling clinical problem.

Depending on the priorities of the individual investigator/practitioner target population, drug formulation and administration as well observation duration would vary significantly. These differences should not be a concern as long the various tradeoffs in the decision-making process are well-identified and addressed in a manner that is consistent to the study question/therapeutic goal. Conversely, study design or treatment scheme decisions based on extraneous constrain would inevitably result in an inadequate intervention. The goal of this manuscript is to discuss these variables in the context to provide the rationale to design future LT3:LT4 combination therapy clinical trials.

TARGET POPULATION

Etiology

Hypothyroidism is a highly prevalent condition (26, 27), but extremely heterogeneous in terms of severity (i.e., degree of endogenous production of thyroid hormone loss), etiology, and comorbidities. In the USA, autoimmune thyroid disease is by far the most common cause of hypothyroidism (frequently detected in the subclinical state), followed by radioiodine ablation for Graves' disease and thyroidectomy. Currently, the vast majority of the prescriptions for LT4 are directed to patients that are affected by subclinical hypothyroidism, which by definition is characterized by residual production of thyroid hormone sufficient to maintain the circulating levels within the normal range. Conversely, individuals who have undergone total thyroidectomy and remnant ablation have no residual endogenous production of thyroid hormone. Clearly, the pathophysiology of the thyroid axis of these two conditions at the extremes of the spectrum of hypothyroidism differs immensely, and it should be taken in consideration when designing a study.

Two diametrically opposed approaches to recruiting the study population have both benefits and drawbacks, which should be carefully evaluated in designing and powering a trial. A recruitment strategy that includes self-described "dissatisfied patients" or "all comers" would be representative of the statistical universe of patients affected by hypothyroidism, and provide an agnostic approach to the question of who could benefit the most from non LT4-only treatment. To achieve statistical power to detect significant differences, such a study would require a population comparable in size (thousands) to the ones performed for diabetes or cardiovascular disease. An important caveat inherent to this approach is that the clinical significance of the study endpoint (*see below*) may not be clearly appreciated by practitioners or lay public. A specific consideration on the strategy of recruiting all comers lay in the diagnosis of hypothyroidism since the sole treatment with thyroid hormone replacement therapy does not constitute a clinical diagnosis, and patients may not recollect the initial TSH level that led to the initiation of treatment. Corrective actions to improve the selection process could include a screening process that takes into account previous documentation of clearly pathologic levels of TSH, or evidence of treatment (surgery or radioactive iodine). In the absence of such evidence, one could use as proxy the presence of anti-TPO antibodies, although this would not equate to lack of endogenous production of thyroid hormone. One could propose to confirm the diagnosis of hypothyroidism by assessing the rise in TSH following discontinuation of the therapy, but this extra step would cause ethical concerns and likely decrease the ability of recruiting study patients.

A conceptually opposite recruitment strategy to the "all comers" consists of developing very strict inclusion criteria, which would provide the best odds to achieve a statistical and clinically relevant outcome by increasing the signal to noise ratio. The specular drawback of this decision would be the risk of excluding patients who may benefit from non-LT4-based therapy since at the present time, the understanding of the "ideal" target population is based on limited observations, or inferred from the pathophysiology of the various forms of hypothyroidism.

Symptomatology

If one would approach the study question from a patient-centered perspective, the primary driver of the decision of switching from LT4 to LT3:LT4 combination or DTE would be purely based on symptoms. This is certainly a valid question, but

TABLE 1 | LT3:LT4 combination therapy studies for the treatment of hypothyroidism.

References	Study design	Patient No.	Type of Hypothyroidism	Drug formulation	1*Outcome	Other outcomes
Bunevicius et al. (9)	Randomized, blinded crossover with two 5-wk periods	26	Autoimmune + postsurgical (11+15)	LT4 at usual dose or minus 50 mcg and adding LT3 at 12.5 mcg with LT4:LT3 ratio 3:1 to 15:1	• Improvement in mood on POMS scale in thyroid cancer patients on combination • Improvement in digital symbol test and visual scan test in thyroid cancer patients on combination therapy	• No change in Beck depression inventory test and Spielberger State-Trait Anxiety Inventory (SSTAI) • serum cholesterol similar in both groups • SHBG and pulse rate higher in combination treatment group
Bunevicius et al. (10)	Randomized, blinded crossover with two 5-wk periods	10	Postsurgical, subtotal thyroidectomy for Graves' disease	LT4 at usual dose or minus 50 mcg and adding LT3 at 10 mcg with LT4:LT3 ratio 5:1 to 10:1	No statistically significant difference in mood, cognitive Scale and hypothyroidism symptoms score	6 patients preferred combination therapy, 2 patients preferred monotherapy and 2 had no preference
Sawka et al. (11)	Randomized, blinded controlled, 15 week	40	Autoimmune	20 patients LT4 only and 20 LT4+T3 (Pre-study LT4 dose reduced to 50% and LT3 added 12.5 mcg twice daily	No statistically significant difference in symptoms, mood, depression scores or general well-being scores	
Clyde et al. (12)	Randomized, double blind, placebo controlled,4 months trial	44	Autoimmune + postablative + postsurgical +post EBRT (39+10+2+1)	LT4 monotherapy usual dose (13) vs. reduced dose of LT4 (usual-50 mcg)+LT37.5 mcg twice daily. Doses adjusted every 5 weeks	• No difference in TSH at 4 months • No differences in QOL assessment between treatment,1/13 neuro cognitive assessment significantly different in favor of monotherapy	
Walsh et al. (14)	Randomized, blinded controlled, 2-group crossover with two 10-wk periods, separated by 4 week of T4 alone	110	Autoimmune+ postablative + postsurgical (94+4+12)	LT4 at usual dose followed by LT4+LT3 ($n = 56$). Group 2 had reverse order ($n = 54$). For combined treatment, L4 usual dose minus 50 mcg and adding LT3 at 10 mcg	• No significant difference in quality of life score • Higher GHQ28 score indicating worse psychological well-being in combination group • No difference in cognitive scores	No difference in treatment satisfaction scores
Siegmund et al. (15)	Randomized, blinded crossover with two 12-wk periods	23	Postsurgical + autoimmine (21+2)	LT4 at same dose or 95% LT4 with 5% substituted as LT3 equivalent to an absorbed molar mixture of 14:1. After 6 weeks, dose was adjusted	• TSH significantly lower in the combination therapy • No significant change in mood, cognition and general well-being scores	1 person had atrial fibrillation on combination with suppressed TSH
Appelhof et al. (16)	Randomized, controlled 15 week	130	Autoimmune	LT4 alone (17) vs. LT4:LT3 ($n = 46$) 10:1 vs. LT4:LT3 ($n = 47$) 5:1, combination adjusted at 5 weeks	Patient preferred combination therapy. Preference for treatment as –LT4 alone 25%, LT4:LT3 10:1 41%, LT4:LT3 5:1 42%	• TSH levels lower in patients receiving combination • Significant weight loss in LT4:LT3 5:1 group (1.7 kg) • No difference in mood, and in general well-being scores
Escobar-Morreale et al. (18)	Randomized, double blind, crossover design with three 8-wk periods	26	Autoimmune + postablation for Graves or MNG (23+5)	• 14 patients received LT4 100 mcg alone for 8 week, 13 patients then LT4 75MCG+LT3 5 mcg for 8 weeks, followed by LT4 87.5 mcg+LT3 7.5 mcg ($n = 12$) • 14 patients received LT4 75MCG+LT3 5 mcg for 8 weeks, followed by LT4 100 mcg 8 weeks, followed by LT4 87.5 mcg+LT3 7.5 mcg for 8 weeks	• No difference in LT4 and LT4+LT3 75+5 mcg group in POMS, on the Digit Symbol Substitution Test, or on the Visual Scanning Test. Slight improvement in the backward and total scores of the Digit Span Test • No difference between the LT4+LT3 87.5+7.5 mcg group and previous treatment in terms of POMS or the Digit Span Test. Better performance Digit Symbol Substitution Test and the visual scanning test	12 patients preferred LT4+LT3 75+5 mcg, 2 preferred LT4, 6 preferred LT4+LT3 87.5+7.5 mcg, 6 had no preference

(Continued)

TABLE 1 | Continued

References	Study design	Patient No.	Type of Hypothyroidism	Drug formulation	1*Outcome	Other outcomes
Rodriguez et al. (19)	Randomized, blinded crossover with two 6-wk periods	27	Autoimmune+ postablative + postsurgical (23+4+3)	LT4 at usual dose or minus 50 mcg and adding LT3 at 10 mcg with LT4:LT3 ratio 5:1	No difference in fatigue score between groups	• No difference in depression score, hypothyroid symptoms and TSH • 7 preferred LT4, 12 preferred LT4 +LT3, 8 had no preference
Saravanan et al. (20)	randomized, parallel group, controlled 12 months trial	697 (573 analyzed)	• Not mentioned • Excluded thyroid cancer patients	LT4 at usual dose ($n = 353$) or minus 50 mcg and adding LT3 at 10 mcg($n = 344$)	Improvements in GHQ caseness at 3 months but not GHQ Likert scores and the initial differences were lost at 12 months	Improvements in GHQ Hospital Anxiety and Depression questionnaire-anxiety scores at 3 months but Hospital Anxiety and Depression questionnaire-depression, thyroid symptoms, or visual analog scales of mood and the initial differences were lost at 12 months
Valizadeh et al. (21)	randomized, double blind, parallel group, 4 months trial	71	Autoimmune + postablative +postsurgical (46+12+2)	LT4 at usual dose ($n = 35$) or minus 50 mcg and adding LT3 at 6.25 mcg BID ($n = 34$). Typical LT4:LT3 4:1,LT4 doses adjusted after 1 month to normalize TSH	The overall score of GHQ-28 was not significantly different between LT4 and combined LT4/LT3 groups. Of the four subscales of the GHQ-28, the only significant difference was observed in the mean score of anxiety/insomnia. In favor of combined LT4+LT3 group	
Nygaard et al. (22)	Randomized, blinded crossover with two 12-wk periods	59	Autoimmune	LT4 at usual dose or minus 50 mcg and adding LT3 at 20 mcg with mean LT4:LT3 ratio 4:1.dose of LT4 adjusted every 4 weeks	• Significant beneficial effect on QOL and depression score (7/11 measures) in favor of combination therapy • Significant beneficial placebo effect with t4 therapy in 10/11 measures	49% preferred combination, 15% preferred LT4, 35% had no preference
Fadeyev et al. (23)	Randomized, controlled, non-blinded 6 month study	36	• Not mentioned • Newly diagnosed patients	LT4 at dose 1.6 mcg/kg ($n = 20$) vs. LT4 dose 166 mcg/kg-25 mcg +LT3 12.5 mcg daily	• No difference in TSH • Total and LDL cholesterol significantly lower in combination group	No difference in preference for treatment for either regimen
Kaminski et al. (13)	Randomized, blinded crossover with two 8-wk periods	32	Autoimmune + postablative + postsurgical (23+3+6)	• Patients were on stable dose of 125 or 150 mcg LT4 before entering study • LT4 at usual dose or LT4 75 mcg+LT3 15 mcg with LT4:LT3 ratio 5:1	• Free t4 levels were significantly lower and resting HR slightly higher with combination vs. monotherapy • No changes observed in QOL questionnaire, lipids, BMI	
Krysiak et al. (24)	Quasiblind, randomized	39	Post-hemithyroidectomy, females only with symptoms of hypothyroidism	Usual levothyroxine dose vs. LT4/LT3 combination in ratio 5:1	• Combination therapy had beneficial effect on 2/6 domains in female sexual function index (FSFI) • No difference in depression score between treatment groups	No difference in TSH between groups.

of extreme complexity from the technical perspective. In fact, setting aside the diagnosis of hypothyroidism and its severity (*discussed above*), researchers are faced with the heterogeneity of the manifestations of hypothyroidism, which are a panoply of aspecific signs and symptoms that have significant overlap with non-affected patients and with patients affected by other conditions (28). Importantly, very often Hashimoto's thyroiditis disease is associated with other autoimmune conditions that are also associated with aspecific symptoms, creating a significant confounder in the attribution of the symptoms to hypothyroidism. Another relevant confounder is represented by the slow and progressive onset of thyroid failure, which makes it extremely difficult from the patients' perspective to attribute with certainty symptoms to hypothyroidism rather than to other co-morbidities or to the aging process. Moreover, given the chronicity of this condition, it is challenging to discern from the patient's perspective the difference of the state of health prior to the development of hypothyroidism from an aspirational state of well-being. All these confounders play a critical role in selecting the study outcome and measures, which will be addressed in details in the next section.

By limiting the study population to specific causes of hypothyroidism, one could significantly reduce the heterogeneity of the sample population, increasing the power of the study to detect statistically significant differences in the primary outcome of the study. To this end, the most appealing strategy would be to limit the recruitment to thyroidectomized patients. The two major advantages of this choice reside in the fact that patients who have undergone total thyroidectomy have by definition no residual thyroid hormone production, and transition acutely from a state of euthyroidism to being entirely dependent from exogenous thyroid hormone therapy. This approach would work well if the goal of the study resides in assessing laboratory-based endpoints. On the other hand, while patients who have undergone total thyroidectomy represent an ideal "experimental platform" to study the effects of thyroid hormone replacement therapy, they are a small minority of patients affected by hypothyroidism, and thus are not representative of the condition.

Genetic Polymorphisms

Genetic background plays an important role in the response to drugs, and several genetic polymorphisms in the thyroid hormone metabolism and signaling (**Table 2**) have been associated with changes in thyroid hormone levels and to some degree with response to therapy (29–35).

The discovery of the Thr92Ala polymorphism of the type 2 deiodinase gene (29), its association with subtle changes in thyroid hormone homeostasis (36), quality of life indices, and response to LT3:LT4 combination therapy (32) has prompted enthusiasm as a potential explanation of patient dissatisfaction (37). No study has prospectively analyzed the contribution of this polymorphism to quality of life or preference to LT3:LT4 combination therapy. Conversely, a prospective pharmacogenomic study carried out with healthy volunteers has demonstrated subtle differences in the pituitary thyroid axis response to TRH injection (38), in keeping with the hypothesis that the Ala 92 allele contributes to a decreased availability of T3 at the end organ tissues. The high prevalence (0.35) of the minor Ala92 allele across ethnicities (29) assures that ∼50% of a random sample will be a carrier of this polymorphism in either homozygous or heterozygous. Thus, it is conceivable to design a study with selective recruitment based on the genotype, or alternatively allowing for a Mendelian randomization (i.e., enabling to perform a secondary analysis while knowing in advance the predicted allocation of the population). Although appealing, this strategy does not take in account other common polymorphisms in the deiodinase or other genes that could affect the thyroid hormone signaling. Indeed, we demonstrated a modulatory effect of the −258A/G D2, another common polymorphism of the type 2 deiodinase gene on the pituitary thyroid axis response to TRH injection (39). Paradoxically, this genetic variant (which is not associated with the Thr92Ala) is associated with increased sensitivity of the pituitary thyroid axis to the TRH stimulation. This finding suggests that the −258A/G D2 confers an increased deiodinase activity, likely exerted by removing a suppressor element in the promoter of the gene (40). Collectively, the data available indicate that the modulation of the thyroid axis, and by extension its response to exogenous replacement therapy, is exerted by a complex polygenic mechanism and no single genetic variant plays a prominent role. The effects of less common genetic variants may be clinically relevant when associated as haplotype in combination with other common variants, but designing a prospective study with such pharmacogenomic recruitment strategy would be extremely challenging. A more realistic strategy would be based on performing exploratory analyses after the completion of the data accrual.

Serum T3 Levels

LT3:LT4 combination therapy or DTE administration should supply the exogenous T3 whose production is lost due to thyroid failure. Thus, targeting the recruitment to individuals with serum T3 levels at the low end or below normal limits is a viable possibility to increase the chances of detecting a statistical and clinically significant difference between treatment arms. This strategy has a clear theoretical appeal, and does not rely on assumptions about the relative role of specific genetic variants. On the other hand, there is no clear correlation between serum T3 levels and symptoms in hypothyroidism. Moreover, T3 levels are quite variable and exquisitely sensitive to changes in the health and nutrition status (41, 42). This could result in a major confounder in the selection of the study population. A corrective action to minimize this confounder would be to allow for a run-in period (with adequate replacement therapy and target TSH) aimed to confirm that the T3 levels are low independent from intervening pathologies. Additionally, individuals who have undergone weight loss, even remotely, should not be recruited because T3 levels remain low months and years following weight reduction (43). Similarly, chronic comorbidities or therapy with amiodarone, propranolol, or steroids can cause lowering of the serum T3 concentrations. While on the one hand, including these patients may tilt the study toward a more "real-life" effectiveness trial, on the other hand, these comorbidities and

TABLE 2 | Common genetic polymorphisms (Single Nucleotide Polymorphisms-SNP) associated with thyroid hormone axis and response to therapy.

References	Polymorphism(s)	Gene	Function	Finding	Notes
Mentuccia et al. (29) Panicker et al. (32)	Thr92Ala rs225014	Type 2 deiodinase (DIO2)	T4→ T3 conversion	Decreased activity, associated with improved response to LT3:LT4 therapy	Synergistic effects with other polymorphisms
Peeters et al. (30)	Asp727Glu rs1991517	TSH receptor (TSH-R)	TSH receptor	Lower TSH levels, no changes in thyroid hormone	
Peeters et al. (30)	C785T rs11206244	Type 1 deiodinase (DIO 1)	rT3→ T2 T4→ T3 conversion	Correlation between the T allele and rT3 levels	Interpreted as loss of function
Peeters et al. (30)	A1814G rs12095080	Type 1 deiodinase (DIO1)	rT3→ T2 T4→ T3conversion	Correlation between the G allele and rT3 levels	Interpreted as gain of function
Peeters et al. (31)	D2-ORFa-Gly3Asp −258 A/G rs12885300	Type 2 deiodinase (DIO2)	T4→ T3 conversion	Increased activity, changes in serum T3:FT4 ratio	
Medici et al. (33)	rs1382879 rs2046045 rs9687206 rs12515498 rs832790 rs1351283 rs989758	Phosphodiesterase 8B (PDE8B)	TSH signal transduction	Association with higher TSH levels	Medici et al. (33)
Medici et al. (33)	rs7714529	Phosphodiesterase 8B (PDE8B)	TSH signal transduction	Association with lower TSH levels	
Roef et al. (34)	rs5937843	Monocarboxylate transporter 8 (MCT8)	T3 cell membrane transporter	inverse association with FT4 concentrations	
Roef et al. (34)	rs6647476	Monocarboxylate transporter 8 (MCT8)	T3 cell membrane transporter	Inverse association with FT3 levels	
Carlé et al. (35)	rs17606253	Monocarboxylate transporter 10 (MCT10)	T3 cell membrane transporter	Carriers of both rs17606253 and rs225014 tend to prefer LT3:LT4 therapy	Synergistic effects with Thr92Ala variant

confounding factors may reduce the power of the study and make the interpretation of the findings challenging.

ENDPOINTS

The selection of study endpoint(s) is one of the most consequential decisions in the development of a successful clinical trial able to prove or disprove the efficacy and effectiveness of LT3:LT4 or DTE therapies. Just as in the selection of the study population, the choice of primary endpoint will be the result of a series of tradeoffs between scientific rigor, feasibility, and clinical relevance of the findings. In general terms, each clinical study stakes its endpoint "goalpost" to the measure that best reflects the efficacy of the drug tested. This is a well-established practice in cardiovascular medications, and more recently in glucose-lowering drugs studies where improvement in cardiovascular disease mortality is considered the ultimate primary outcome. Quite often, budgets and feasibility constrain limit the choice of primary endpoint to "second-best," which is a valid proxy for the stated goals (e.g., composite cardiovascular endpoints). This approach is extremely complicated in the space of thyroid hormone replacement therapy where there is objective difficulty in determining the specificity of the symptoms, and the laboratory-based outcomes are affected by other biological factors. Below, we include some approaches to selecting the study endpoint that we believe are important in the decision-making process.

Symptoms-Based Endpoints

The most common complaints attributed by patients to hypothyroidism concern quality of life. Fatigue, difficulties in concentrating, "mental fog," and depression are the most common descriptors. Unfortunately, they are highly aspecific (28), affected by comorbidities and life stressors, and their quantification is challenging. Over the years, various general and thyroid disease-specific quality of life (QoL) instruments have been generated to interrogate the prevalence and to quantify the symptoms associated with thyroid disorders (3, 12, 44, 45). More recently, the ThyPRO has shown to be valid (46) and has been successfully used in large studies (47). Major advantages of disease-specific QoL instruments reside in the thorough validation process and in the possibility of deriving numerical scores that allow for detection of statistical differences among groups. The disadvantage of this approach resides in the variability of the symptoms among patients (28). In other words, while the plurality of patients complains of an aggregate of symptoms captured by the QoL instrument, at the individual level the symptom/sign that is debilitating may not have sufficient weight. Moreover, it is difficult to translate the clinical relevance of differences, albeit statistically

significant ones, in QoL instruments to the day-to-day life experience of the individual patient. Therefore, the translation to clinical practice of the findings of a scientifically rigorous study could be challenging. A possible alternative strategy, not yet formally studied, would be to query the individual patient at the time of enrollment about the symptom which s/he finds more debilitating and attributes to hypothyroidism. Such symptoms (by default different among study participants) could be assessed on a visual analog scale, and then its differences could be evaluated during the study.

Signs and Laboratory-Based Endpoints

Thyroid hormones affect virtually all organ systems, and there is a panoply of markers of thyroid hormone action which could be utilized as endpoints for a clinical trial. Body weight is probably the most widely recognized proxy for thyroid hormone action, even if the association between weight gain and hypothyroidism is at best tenuous. Importantly, weight gain is one of the most important drivers of dissatisfaction among patients affected by hypothyroidism (3, 4, 17, 45, 48) and conversely, weight loss was the greatest reason to declare satisfaction with the treatments among patients who preferred combination therapy or DTE on clinical trials (9, 10, 18, 22, 25). Moreover, a crossover study indicated that therapy with LT3 alone was associated with significant weight loss (49). Weight changes should thus be part of the measures captured in clinical trials, either as a secondary endpoint or as an explanatory variable for satisfaction. Extreme care should be taken in recording weight, including the use of standardized clothing and well-defined standard operating procedures to increase the reliability of the measurements. Depending on the study design, additional physiologic measurements may be considered depending on whether the study is more geared toward symptoms or exploration of the pathophysiology of the thyroid hormone replacement therapy. To this end, the measurement of energy expenditure by means of indirect calorimeter would provide important information (50). While hood calorimeters (metabolic carts) are commonly available, their sensitivity may not be sufficient to capture variations in energy expenditure, which are associated with small changes in body weight (49). Moreover, these systems do not capture all the components of energy expenditure. A more comprehensive strategy would be using extended recordings in whole room indirect calorimeters (51), but only few institutions have these instruments. One can envision, though, their use in proof of concept studies or in a nested study in the context of a large multicenter trial.

Lipid metabolism is directly affected by thyroid hormone action (52), and although extremely variable within the population, the intraindividual variation of serum lipids is remarkably small, making changes in serum lipids a reliable (and economical) variable which would be easy to capture. Although the cardiovascular system is an important target of thyroid hormone action (53, 54), a thorough assessment of the structural and functional changes would require dedicated resources beyond the scope of a clinical trial large enough to demonstrate effectiveness (i.e., patient-centered) results. It is conceivable nonetheless that proof of concept studies or nested studies within multicenter trials could address this topic. Although unlikely to show significant differences, blood pressure and heart rate should be recorded according to a well-defined standard operating procedure protocol to provide important information on potential safety signals. From a purely pathophysiologic exploration, additional targets of thyroid hormone action (e.g., body composition, sex hormone binding globulin, angiotensin converting enzyme, etc.) could be captured.

FORMULATIONS AND DOSING

The theory behind LT3:LT4 combination therapy or DTE administration is the replacement of endogenous T3 loss because of the development of hypothyroidism and/or due to a hypothetical deficit in peripheral conversion of exogenously-administered LT4 into T3 at the tissue target of the thyroid hormone action. Alternatively, one could aim to exploit the pharmacologic effects of T3 by providing higher doses than the estimated production from the thyroid. Irrespective, pharmacokinetics of LT3 and DTE, potential risks of overdosing, and feasibility are important determinants of the design of formulations and dosing frequency for a clinical trial.

The estimation of the contribution of the thyroid gland to the pool of circulating T3 suggests that the daily production of T3 is ∼5 mcg/day, while the rest is the result of peripheral conversion of T4 (8). Thus, if the therapeutic goal is the replacement of this component, the formulations of LT3 in a LT3:LT4 combination should approximate this dose, ideally with adjustments for body weight, or limiting the recruitment to individuals within a specific range of body weight. **Table 1** summarizes the treatment schemes in the LT3;LT4 combination therapy, and shows that with the exception of one trial in which the LT3:LT4 ratio was maintained at 1:14 (15), all the studies have used higher doses of LT3. In this series of articles, Drs. DiStefano and Jonklaas have provided an elegant theoretical modeling to titrate the LT3 dose in LT3:LT4 combination therapy schemes (55). Although appealing, this is not feasible in practice since the costs of individual formulations would be exorbitant. Indeed, in the USA, LT3 formulations are available in 5, 25, and 50 mcg strengths, so realistically the only options are fixed doses multiple of 2.5 mcg (half tablet) with matching placebo or for overencapsulation. Any greater degree of individualization would require a research pharmacy with the ability to produce tablets from bulk material. This is doable in small, proof of concept studies, but it would be highly impractical for large studies.

The pharmacokinetics (PK) characteristics of LT3 provide yet another layer of complexity since this formulation has a much shorter half-life when compared to LT4. Indeed, in our original crossover trial in which we treated hypothyroid patients devoid of endogenous production of thyroid hormone with LT3 or LT4 on a thrice daily administration scheme (56), we noted significant fluctuations of T3 while patients were treated with LT3 (49). Very recently, we formally characterized the PK of LT3 in the absence of endogenous or exogenous T4. The data are best explained by a two-compartment model with a fast distribution phase, and a

much slower elimination phase with two distinct half-lives, 2.3 and 22.9 h, respectively (57). Based on these data, we were also able to generate a mathematical model able to predict the changes in T3 concentration and its fluctuations on various therapeutic schemes. Maintaining the same daily dose of LT3, the average serum concentration would not change between single vs. twice or administration regimens, but its variance between peak and trough would change dramatically. Moreover, we were able to predict the changes in T3 concentrations by adding LT3, either 3.75, 5, or 10 mcg twice daily, while decreasing the hypothetical LT4 dose of 112 mcg by 10, 25, or 50 mcg, respectively in a hypothetical 70 Kg patient. Our modeling indicates that in these scenarios, the mean serum T3 concentration would be increased from a baseline of 93 ng/dl (low end of normal range) by 26, 41, and 82 ng/dl, respectively. Of interest, only the highest LT3 dosing scheme would result in serum T3 concentrations above the upper level of reference, ∼14% of the 24 h period (57). These theoretical data indicate that a twice daily administration scheme is feasible and would result in acceptable variations of serum T3 concentrations. On the other hand, such a scheme would still require a twice daily administration regimen, which is not ideal for a lifelong treatment.

There is general consensus that peaks of serum T3 concentrations may result in acute and potentially lethal toxicity, particularly at the level of the cardiovascular system which would be directly exposed to rapid rise in T3 should the administration be performed on a single daily regimen. This concern is supported by the well-known toxic effects of sustained thyrotoxicosis and, to some degree, by the demonstration of rapid, non-genomic effects of thyroid hormone which are exerted at the level of the vascular endothelium (58, 59). Conversely, it is worth nothing that while the non-genomic effects of thyroid hormone are well-characterized *in vitro*, their clinical relevance is not clear. Of interest, a PK study performed in healthy volunteers with pharmacologic doses of LT3 did not show any measurable change in blood pressure until 5 h following the drug administration (60), indicating that the action of thyroid hormone on the cardiovascular system (limited to blood pressure and heart rate) is mediated by its effect on gene transcription rather than rapid, non-genomic mechanisms. Whether thyroid hormones exert other less evident but potentially clinically relevant non-genomic effects on the cardiovascular system should be explored in detailed studies. This is important because the scientific community would not accept a single daily administration regimen of LT3 (with consequent peaks and throughs) in the absence of a clear demonstration of the safety of this scheme.

STUDY DESIGN

The duration of the study and its design are critical decision points in the development of an adequately powered and internally valid study to prove or disprove the efficacy of LT3:LT4 combination therapy or DTE for the treatment of hypothyroidism. There is clear consensus about the need to perform double blind intervention with an adequate observation period to mitigate placebo effect and to allow for the effects of different formulations of thyroid hormone to exert their actions on the various endpoints, be they anthropometrics, laboratory-based, or QoL (2). Additionally, repeated measures can demonstrate trends or regression to the mean of various endpoints. Considering that thyroid hormone replacement is a lifelong therapy, and that (relative to LT4) a steady state is reached after 6 weeks from the dose adjustment, a long observation period (12 or 24 months) would be ideal. The long duration of the study would also allow for therapy adjustments, and also allow for gathering data on potential toxicity. Conversely, since the study drugs are available in commerce, one can expect a significant attrition during the study, with participants who do not experience the expected improvement being likely to drop out and to request their physicians to prescribe LT3:LT4 combination therapy or DTE. A crossover design is appealing because of the increase in statistical power due to the ability of performing paired analyses (25, 49). This advantage is mitigated by the potential carryover effect of the first study drug on the second treatment, and the risk of loss of data in case of drop out.

DESIGNING THE "IDEAL STUDY"

The lack of definitive data on the efficacy and effectiveness of LT3:LT4 combination (or DTE) for the treatment of hypothyroidism has preempted the professional societies to endorse these therapeutic modalities. This is completely understandable due to the potential for toxicity due to excessive LT3 dosing, and the increased costs and complexities in the treatment schemes when compared to once-a day LT4. There is obvious need of compelling data that could prove or disprove the value of therapies other than LT4 alone. To this end, the stakes in designing such a study are enormous, and the tradeoffs described in the previous sections need to be carefully considered in order to make conscious decisions rather than being forced to compromise to a study that may not have sufficient statistical power or have a primary endpoint that does not sufficiently address the study question. The major decision points researchers (and funding agencies) will need to face (**Figure 1**) are about the primary endpoint and on the dosing and ratio LT3:LT4 formulation or accepting the non-physiologic T3:T4 content of DTE. While there are many validated QoL, it may be challenging correlating the changes in these instruments with the symptoms of the individual patient. Similarly, while changes in laboratory-measured parameters are easily quantifiable, their clinical significance and their relevance to the "dissatisfaction" question could be disputed. Relative to the study drug formulation, the major decision point is whether to aim for a "physiologic" replacement of the T3 lost from lack of thyroid function or to use higher doses to exploit the pharmacologic effects of T3, possibly raising the serum concentrations above the individual's levels before developing hypothyroidism. This latter approach may provide a greater effect size, but conversely could expose patients to untoward toxicity. Likewise, a better understanding of the

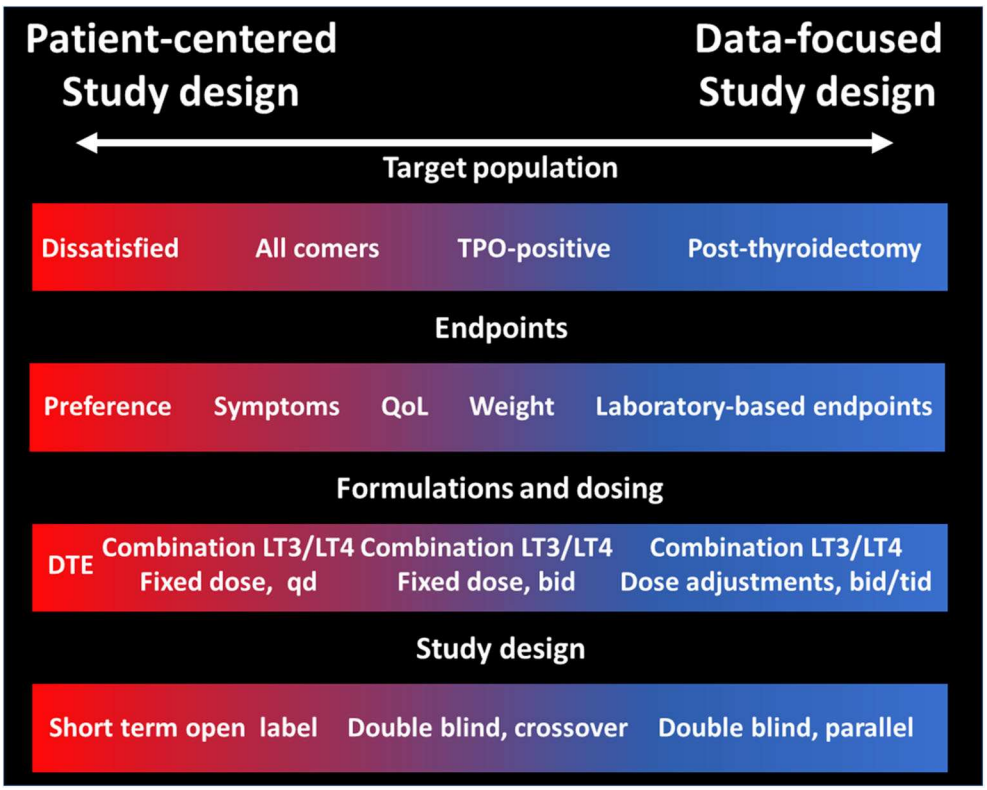

FIGURE 1 | Tradeoffs in study design of LT3:LT4 combination therapy or DTE vs. LT4 alone for the treatment of hypothyroidism. Tradeoffs in four domains of a clinical trial directed to assess the efficacy and effectiveness of therapies other than LT4 alone vs. LT4. The left part of the panels (red) indicates choices more suitable for a patient-centered study, while the right side of the panels indicates choices more suitable for a data-focused study.

clinical relevance (or lack thereof) of rapid, non-genomic effects of T3 could provide the rationale to designing once daily administration schemes which in turn could improve the adherence to the regimen. Small, proof of concept preliminary studies could provide empirical information, avoiding the scientific risk of relying on theoretical modeling, convenience, or experts' opinions.

CONCLUSIONS

Dissatisfaction toward LT4-only therapy among patients affected by hypothyroidism is a common and well-recognized problem. While all the stakeholders (patients, pharmaceutical companies, physicians, professional organizations, and funding agencies) recognize the need of evidence generated from well-conducted studies, there is still lack of consensus about how an "ideal study" would be structured. Clinical investigators should approach the various tradeoffs in the study design with a clear understanding of how these decisions will affect the internal validity of the study, as well as the applicability and relevance of the findings to scientific community and to the patients, who ultimately are the most important stakeholders.

AUTHOR CONTRIBUTIONS

RM reviewed the literature and contributed to the initial manuscript draft. FC designed the manuscript scope, structure, take home message, and wrote the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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