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Clinical Research Article

Novel Biomarkers Reveal Mismatch Between Tissue and Serum Thyroid Hormone Status in Amiodarone-Induced Hyperthyroidism

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Abstract

Context: Serum thyrotropin and thyroid hormone (TH) levels are routine markers of thyroid function. However, their diagnostic performance is limited under special conditions, such as in amiodarone-induced hyperthyroidism (AIH). Such cases would require the assessment of tissue TH action, which is currently unfeasible.

Objective: Development of an approach that determines how well serum parameters are reflected in tissue TH action of patients.

Methods: TH-responsive marker genes were identified from human hair follicles (HFs) with next-generation sequencing, validated by quantitative polymerase chain reaction. A classification model was built with these markers to assess tissue TH action and was deployed on amiodarone-treated patients. The impact of amiodarone on tissue TH action was also studied in thyroid hormone action indicator (THAI) mice.

Results: The classification model was validated and shown to predict tissue TH status of subjects with good performance. Serum- and HF-based TH statuses were concordant in hypothyroid and euthyroid amiodarone-treated patients. In contrast, amiodarone decreased the coincidence of serum-based and HF-based TH statuses in patients with hyperthyroidism, indicating that AIH is not unequivocally associated with tissue hyperthyroidism. This was confirmed in the THAI model, where amiodarone prevented tissue hyperthyroidism in THAI mice despite high serum free thyroxine.

Conclusion: We developed a minimally invasive approach using HF markers to assess tissue TH economy that could complement routine diagnostics in controversial cases. We observed that a substantial proportion of patients with AIH do not develop tissue hyperthyroidism, indicating that amiodarone protects tissues from thyrotoxicosis. Assessing tissue TH action in patients with AIH may be warranted for treatment decisions.

Key Words: thyroid hormone, tissue thyroid hormone economy, tissue biomarker, amiodarone-induced hyperthyroidism

Abbreviations: AIH, amiodarone-induced hyperthyroidism; AMIO, amiodarone-treated individual; BAT, brown adipose tissue; CALIB, not amiodarone–treated calibrator; fT3, free triiodothyronine; fT4, free thyroxine; qPCR, quantitative polymerase chain reaction; RTH, resistance to thyroid hormone; HF, hair follicle; TH, thyroid hormone; THAI, thyroid hormone action indicator; TSH, thyrotropin.

Circulating thyrotropin (TSH) is a hormonal product of the pituitary and part of the central regulatory system of thyroid hormone (TH) economy. Due to the log negative relationship of serum free thyroxine (fT4) and TSH, shifts in fT4 levels are reflected in TSH levels. Therefore, TSH measurement has been the hallmark of a patient's thyroid health for half a century (1).

However, a local regulatory system for TH action resides in our tissues and has critical autonomy for fine-tuning local TH signaling. This machinery makes TH action tissue

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specific (2, 3). Since the discovery of local regulation of TH action, it has been a matter of discussion how well central parameters can characterize tissue TH economy. Serum TSH or TSH and free TH (fTH) levels are good diagnostic tools and serve as representative measures of TH economy in routine diagnostics (4). However, in certain situations, like in a sizeable minority of patients with hypothyroidism supplemented with thyroxin, in patients with resistance to TH alpha (RTHα) or beta (RTHβ) syndrome, and other conditions when the clinical symptoms and serum biochemistry are equivocal, mere serum hormone level–based approaches are questionable.

A patient group in which such controversies are common are patients on amiodarone treatment. Amiodarone is an antiarrhythmic drug with the potential to alter serum TH levels. The first discoveries of amiodarone effects on TH economy recognized serum TH alterations, but all thyroid-related abnormalities could not be explained only by the high iodine content of amiodarone, which in some cases may lead to either hypothyroidism or hyperthyroidism (5). Amiodarone-induced hyperthyroidism (AIH) is characterized by markedly decreased TSH and highly elevated fTH levels. Intriguingly, these laboratory parameters are usually accompanied by relatively mild clinical symptoms of hyperthyroidism or no symptoms at all (5-7). This discrepancy has been studied before, but the underlying mechanisms are still vaguely known. Early investigations focused on altered serum hormone levels and direct interference with nuclear TH signaling (8-12). Interestingly, data suggested a direct connection of TH-related side effects of amiodarone with its therapeutic efficacy proposing a mechanism that the antiarrhythmic effects are based on moderated local TH action in the heart (13). Other important mechanisms described in vitro are the molecular interferences of amiodarone with TH metabolism by potentially impacting members of the deiodinase enzyme family (14, 15), among which most of the available data discuss the impact on type 2 deiodinase. Based on these, amiodarone is a noncompetitive inhibitor of type 2 deiodinase, that is responsible for converting T4 to triiodothyronine (T3) in tissues, suggesting lower peripheral T3 availability if amiodarone is present (5, 16). Another mechanistical explanation arises from various in vitro models claiming that amiodarone is able to mitigate TH transport (17); however, in vivo data on these aforementioned molecular aspects are controversial. Nonetheless, the wide array of amiodarone effects on TH economy makes clinical decision-making more difficult in patients with AIH since routine approaches targeting serum TSH and fTH levels are potentially less informative under such disruptive circumstances (6, 7).

The potential failure of circulating TSH and TH levels to predict tissue TH action in special clinical situations generated a demand for approaches allowing the assessment of TH action at the tissue level. Sampling of most TH target tissues requires invasive intervention, which is a considerable limitation. Here we present a novel hair follicle (HF)–based approach to measure tissue TH action in patients, which we have applied to investigate the effect of amiodarone on tissue TH economy. Furthermore, we tested the effects of amiodarone on tissue TH action in the TH action indicator (THAI) mouse model (18) to validate our human findings and to extend our investigations towards tissues that are difficult to sample from humans.

Materials and Methods

Human Study

Study design and participants

In this cross-sectional, multicenter study, subjects were enrolled as not amiodarone–treated calibrators (CALIBs) or amiodarone-treated individuals (AMIOs). In the CALIB group, HF samples were taken from patients with hypothyroidism or hyperthyroidism at the participating endocrine centers, and euthyroid samples were taken from volunteers with confirmed normal thyroid function and no known evidence of endocrine disorders (healthy controls). Subjects were eligible if they were at least 18 years old, not pregnant and had no known inherited disease, including RTHα and RTHβ syndromes. Enrolled subjects were not stratified for ethnicity, lifestyle, comorbidities, and background of hypothyroidism and hyperthyroidism. In the AMIO group, amiodarone was started at least 4 weeks before HF sampling. All hyperthyroid AMIO patients in the study had type 1 AIH. Besides amiodarone, commonly used medications (taken by more than 10 patients) were beta blockers (54 patients), furosemide (46 patients), anticoagulants or antiplatelet agents (40 patients), angiotensin-converting enzyme inhibitors (37 patients), mineralocorticoid receptor antagonists (36 patients), proton pump inhibitors (30 patients), HMG-CoA reductase inhibitors (25 patients), allopurinol (24 patients), calcium channel blockers (23 patients), benzodiazepines (19 patients), indapamide (16 patients), angiotensin II receptor antagonists (14 patients), metformin (14 patients), and trimetazidine (13 patients). Figure 1A shows a flowchart of enrollment and exclusions.

Serum and concomitant HF samples were taken the same day, preferably at the same time. Serum TSH, fT4, and fT3 were measured with immunoassays routinely used by the participating centers. Subjects were categorized as hypothyroid, euthyroid, or hyperthyroid based on their TSH and fT4 values.

Collection of human hair follicles and RNA isolation

Ten single hairs were pulled from the subject's vertex skin. The HFs were cut from each hair. HF samples were placed immediately after collection in Eppendorf tubes containing 100 to 300 µL of Arcturus PicoPure kit extraction buffer and stored at −20 °C until used. Total RNA was isolated with the Arcturus PicoPure RNA isolation kit (Applied Biosystems) according to the manufacturers instruction with the following modification. After RNA extraction, instead of the instructed amount, the same volume of 70% ethanol was added as the volume of extraction buffer in which the sample was lysed in. On column DNAse treatment was performed on all samples. RNA samples were kept at −80 °C until use for gene expression analysis.

Next-generation sequencing

Five hypothyroid, euthyroid, and hyperthyroid CALIB HF samples of the best RNA quality among the available candidate samples were selected for next-generation sequencing in order to record the TH-sensitive transcriptome of this tissue. We aimed to use these data to identify TH-sensitive genes as biomarker candidates.

Quantity and quality of the purified RNAs were checked at first with a photometric method. According to the

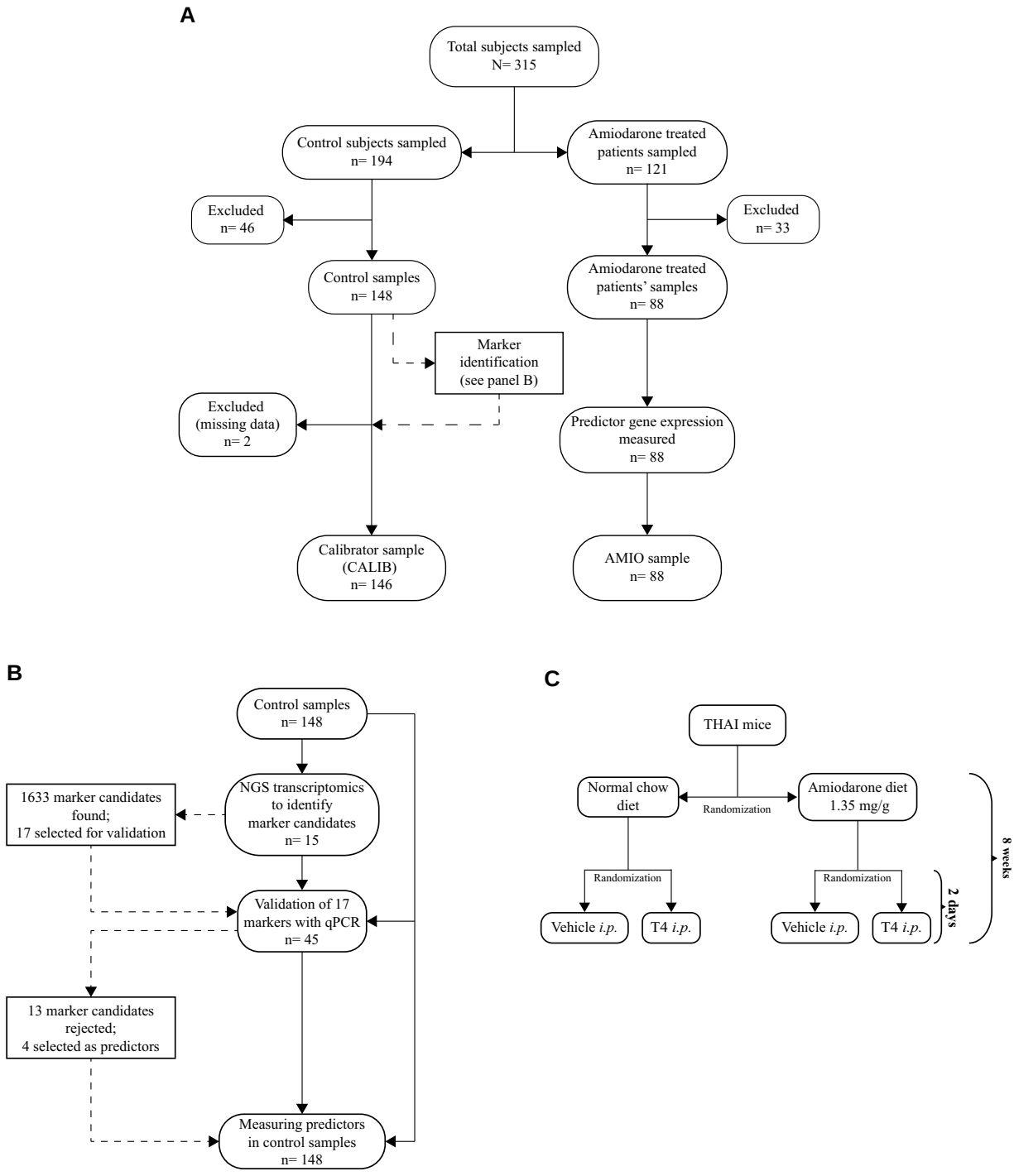

Figure 1. Flowcharts and experimental designs of the studies. (A) Flowchart of the human study. Exclusions were applied for not meeting all inclusion criteria for the given group. (B) Flowchart of marker gene identification. Continuous lines mark subjects, sequenced lines mark genes. (C) Experimental design of the animal studies. Male THAI mice were treated with amiodarone hydrochloride in the diet (1.35 mg/g chow) for 8 weeks and received T4 intraperitoneally on the last 2 days (166.7 ng/bwg/day T4 in the lungs and BAT study, 250 ng/bwg/day in the heart study). Abbreviations: N, population of investigated subjects; n, subpopulation of subjects; NGS, next-generation sequencing; qPCR, quantitative polymerase chain reaction; T4, thyroxine; THAI, thyroid hormone action indicator mouse.

manufacturer's instructions, the RNA concentration and A260/280 ratio were measured with the DeNovix spectrophotometer (DeNovix, Inc., Wilmington, DE, USA).

Then the total RNA sample was subjected to a quality check on an Agilent BioAnalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA) using Eukaryotic Total RNA Nano Kit according to the manufacturer's protocol. Samples with RNA integrity number value >7 were accepted for library preparation process.

High throughput mRNA sequencing analysis was performed on the Illumina sequencing platform (Illumina, Inc., San Diego, CA, USA) by UD-GenoMed Medical Genomic Technologies Ltd (Debrecen, Hungary) to obtain global transcriptome data.

Table 1. List of Taqman gene expression assays

Species	Gene symbol	Gene name	Assay ID
Human	*Bmp2*	bone morphogenic protein 2	Hs00154192_m1
	Col7a1	collagen type VII alpha 1 chain	Hs00164310_m1
	Daam2	disheveled associated activator of morphogenesis 2	Hs00322497_m1
	Entpd8	ectonucleoside triphosphate diphosphohydrolase 8	Hs01368600_g1
	Fzd10	frizzled class receptor 10	Hs04999826_s1
	Gapdh	glyceraldehyde-3-phosphate dehydrogenase	Hs02758991_g1
	H3c1	H3 clustered histone 1	Hs00543854_s1
	Hprt1	hypoxanthine phosphoribosyltransferase 1	Hs02800695_m1
	Insig1	insulin induced gene 1	Hs00356479_g1
	Itgb4	integrin subunit beta 4	Hs00236216_m1
	Kplce	KPRP N-terminal and LCE C-terminal like protein	Hs01104142_s1
	Krt16	keratin 16	Hs00373910_g1
	Krtap9-7	keratin associated protein 9-7	Hs04935058_gH
	Mypn	myopalladin	Hs00261515_m1
	Pgf	placental growth factor	Hs00182176_m1
	Plaat1	phospholipase A and acyltransferase 1	Hs01082527_m1
	Prss27	serine protease 27	Hs01029754_m1
	Tgfb2	transforming growth factor beta 2	Hs00234244_m1
	Ucn2	urocortin 2	Hs00264218_s1
Mouse	*dCpG Luc*	modified luciferase	AIY9ZTZ
	Hprt1	hypoxanthine guanine phosphoribosyl transferase	Mm01545399_m1

Table 2. Characteristics of human subjects

	Hypothyroid	Euthyroid	Hyperthyroid	All subjects
CALIB				
Female	29 (67%)	32 (53%)	33 (73%)	94 (64%)
Male	14 (33%)	28 (47%)	12 (27%)	54 (36%)
Age (years)	47 (36-59)	47 (33.5-59)	37 (30-52)	43 (33-58)
TSH (mU/L)	71.8 ± 28.7	1.97 ± 0.94	0.0063 ± 0.0075	21.7 ± 35.7
fT4 (pM)	4.07 ± 3.22	15.7 ± 2.31	60.3 ± 24.7	25.9 ± 27.1
fT3 (pM)	1.88 ± 1.19	4.95 ± 0.55	25.7 ± 12.7	9.9 ± 12.0
AMIO				
Female	9 (43%)	18 (40%)	8 (36%)	35 (40%)
Male	12 (57%)	27 (60%)	14 (64%)	53 (60%)
Age (years)	68 (65-75)	70 (62-73)	63.5 (60-70)	68 (62-73)
TSH (mU/L)	23.2 ± 30.44	2.14 ± 1.07	0.011 ± 0.022	6.92 ± 17.71
fT4 (pM)	15.9 ± 7.29	21.0 ± 4.61	53.7 ± 23.5	26.8 ± 16.5
fT3 (pM)	3.93 ± 1.31	3.62 ± 1.12	8.97 ± 5.08	4.84 ± 3.32

Data are n (%) for male and female; age shown as median with interquartile range; hormone levels shown as mean ± SD.
Abbreviations: CALIB, not amiodarone–treated calibrator; AMIO, amiodarone-treated individual; TSH, serum thyrotropin; fT4, serum free thyroxine; fT3, serum free triiodothyronine.

RNA-Seq libraries were prepared from total RNA using Ultra II RNA sample prep kit (New England BioLabs). Briefly, poly-A RNAs were captured by oligo-dT–conjugated magnetic beads then the mRNAs were eluted and fragmented at 94 °C. The first strand of cDNA was generated by random priming reverse transcription, and after the second strand synthesis step, double-stranded cDNA was generated. After repairing ends, A-tailing, and adapter ligation steps, adapter ligated fragments were amplified in enrichment polymerase chain reaction (PCR), and finally sequencing libraries were generated. Sequencing runs were executed on Illumina NextSeq 500 instrument using single-end 75 cycles sequencing.

Raw sequencing data (fastq) was aligned to human reference genome version GRCh38 using HISAT2 algorithm, and BAM files were generated. Downstream analysis was performed using StrandNGS software (www.strand-ngs.com).

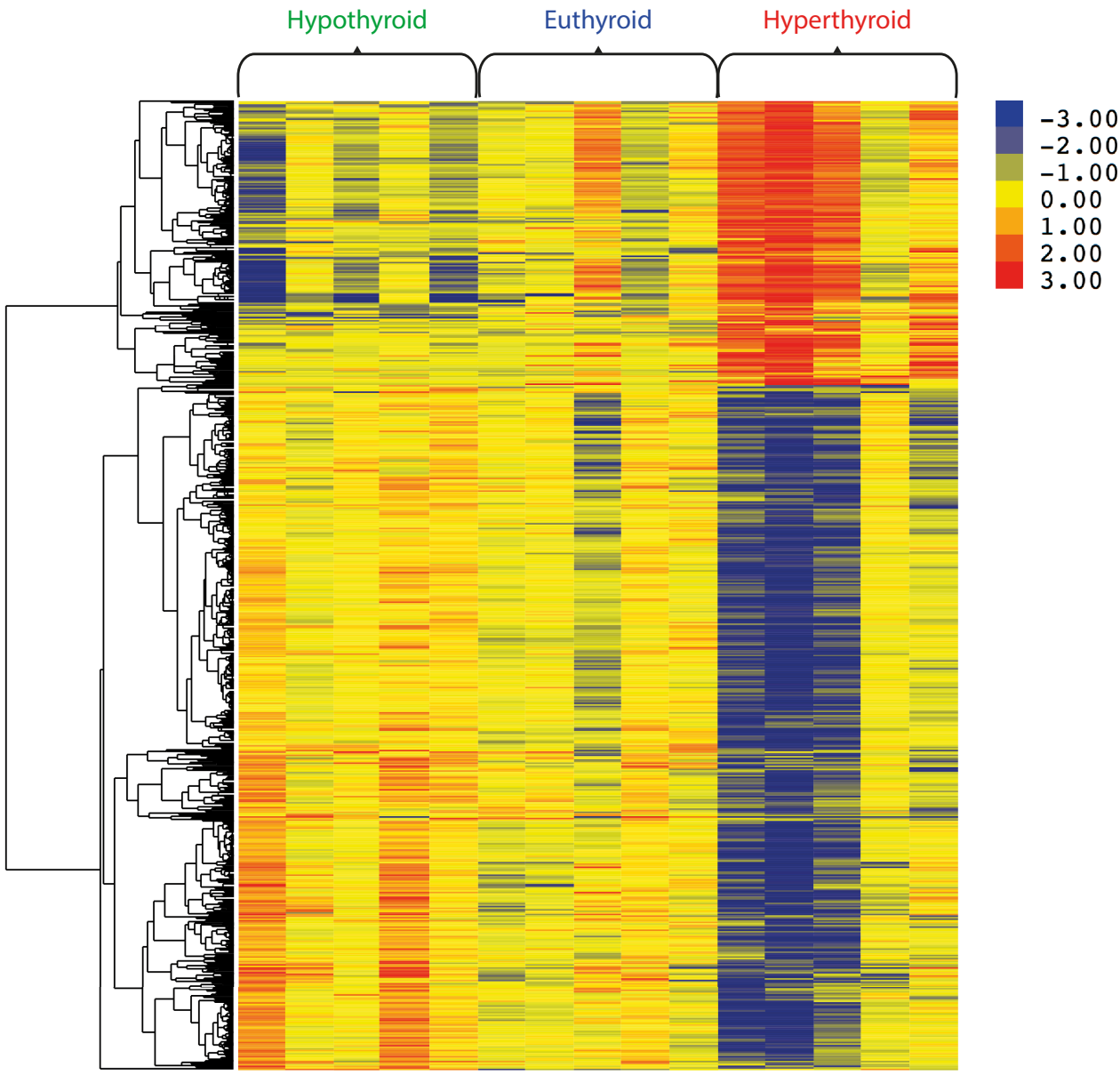

Figure 2. Heat map of hair follicle transcriptomics. Heat map of differentially expressed transcripts of hypothyroid, euthyroid, and hyperthyroid hair follicles. n = 5/group.

BAM files were imported into the software, DESeq algorithm was used for normalization. A Z-test with Benjamini–Hochberg FDR was used to determine differentially expressed genes between conditions.

To generate the heatmaps, Cluster 3.0 (https://www.encodeproject.org/software/cluster/) and TreeView3 (https://bitbucket.org/TreeView3Dev/treeview3/src/master/) applications were used.

Transcriptomes have been deposited to NCBI (bioproject: PRJNA1073747, release date: 02/02/26).

Taqman PCR on tissue biomarker candidates

A high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific) was used to reverse transcribe 14 μL of undiluted total RNA isolate. Then, cDNA concentration was measured with Qubit ssDNA assay kit (Invitrogen). All cDNA was diluted to the same concentration, and 10 ng of cDNA was used in Taqman reactions of the following transcripts: *Bmp2*, *Col7a1*, *Fzd10*, *Insig1*, *Itgb4*, *Kplce*, *Krt16*, *Krtap9-7*, *Prss27*, and *Tgfb2*. *Gapdh* and *Hprt1* were used as reference genes that did not show systemic variability under the study conditions. Preamp master mix (Applied Biosystems) was used to enhance detection of the following transcripts: *Daam2*, *Entpd8*, *H3c1*, *Mypn*, *Pgf*, *Plaat1*, and *Ucn2*. Preamplification and the following Taqman reactions were assembled as instructed in the preamplification manual. All Taqman reactions were assayed on ViiA7 instrument (Applied Biosystems). All data were analyzed as dCt values. PCR efficiency of all Taqman assays used is 1 by design. A list of Taqman assays can be found in Table 1 (Thermo Fisher Scientific).

Statistical analysis of human data

Data were organized with Microsoft Excel. Statistical analysis of human data was done with Tibco STATISTICA v14 and SPSS v23. Both types of software yielded the same result. GraphPad Prism 9 and Adobe Illustrator were used

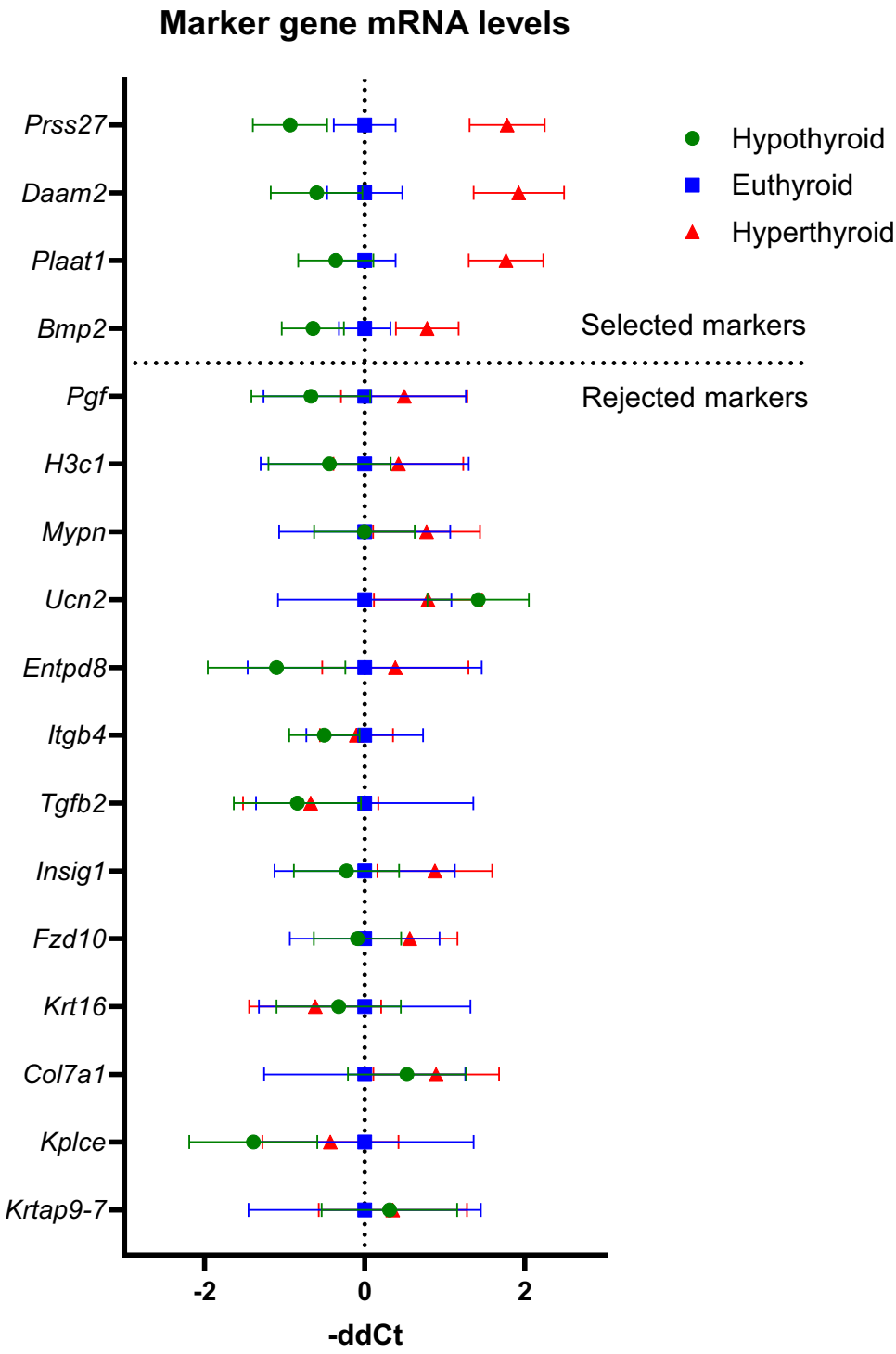

Figure 3. Validated biomarker candidates for tissue TH action in human hair follicles of the CALIB sample. Seventeen biomarker candidates were chosen based on transcriptome data to be validated by Taqman qPCR. Figure shows expression levels of marker genes as mean ± 95% CI estimated with analysis of covariance. One unit increase in –ddCt means a 2-fold increase in gene expression. The euthyroid group was the reference group. N (accepted markers) = 148, n (rejected markers) = 45. Abbreviations: *Prss27*, serine protease 27; *Daam2*, disheveled associated activator of morphogenesis 2; *Plaat1*, phospholipase A and acyltransferase 1; *Bmp2*, bone morphogenic protein 2; *Pgf*, placental growth factor; *H3c1*, H3 clustered histone 1; *Mypn*, myopalladin; *Ucn2*, urocortin 2; *Entpd8*, ectonucleoside triphosphate diphosphohydrolase 8; *Itgb4*, integrin subunit beta 4; *Tgfb2*, transforming growth factor beta 2; *Insig1*, insulin induced gene 1; *Fzd10*, frizzled class receptor 10; *Krt16*, keratin 16; *Col7a1*, collagen type VII alpha 1 chain; *Kplce*, KPRP N-terminal and LCE C-terminal like protein; *Krtap9-7*, keratin associated protein 9-7; mRNA, messenger RNA.

to create figures. In order to make figures more comprehensible, gene expression data were normalized to the mean of the euthyroid group (ddCt) and multiplied by minus 1 (−ddCt). One unit increase in –ddCt means a 2-fold increase in gene expression. The biomarker identification process is outlined in Fig. 1B.

Analysis of covariance was used to compare gene expression levels of hypothyroid, euthyroid, and hyperthyroid

Table 3. Parameter estimates and statistics of prediction model

		Estimate	SE	Wald statistic	Lower CI 95%	Upper CI 95%	*P*
Predicting hyperthyroidism	Intercept	−0.256	0.783	0.107	−1.790	1.278	.74
	Marker genes	−0.727	0.180	16.407	−1.079	−0.375	.0001
	Sex	0.256	0.497	0.266	−0.717	1.229	.60
	Age	−0.012	0.016	0.568	−0.043	0.019	.45
Predicting hypothyroidism	Intercept	−1.283	0.758	2.867	−2.768	0.202	.09
	Marker genes	0.491	0.194	6.392	0.110	0.872	.011
	Sex	0.869	0.464	3.509	−0.040	1.778	.061
	Age	0.001	0.014	0.007	−0.027	0.029	.93

Parameter estimates and statistics of the prediction model for classifying subjects into hypothyroid, euthyroid, or hyperthyroid based on novel tissue markers. The euthyroid group served as reference group.

Table 4. Confusion matrix of prediction model on CALIB sample

	Predicted hypothyroid	Predicted euthyroid	Predicted hyperthyroid
Observed hypothyroid	20	17	5
Observed euthyroid	12	39	9
Observed hyperthyroid	1	19	24

Confusion matrix of calibrator sample after deploying prediction model for classifying subjects into hypothyroid, euthyroid, or hyperthyroid based on novel tissue markers. Observed means serum-based, predicted means tissue marker-based categorization.

subject groups in the CALIB sample with age (in years) and sex as covariates. Sex was coded as male = 0 and female = 1. Preliminary analysis was conducted on all 17 marker candidates on a subset of samples (n = 45). Four markers, *Bmp2*, *Daam2*, *Prss27*, and *Plaat1*, were chosen and analyzed in all cases based on this analysis. Two samples had incomplete measurements, leading to missing data, and had to be excluded from subsequent analyses.

The expression patterns of the selected markers showed a high correlation with each other. Therefore, principal component analysis was conducted to combine markers into 1 variable. The Kaiser method was applied, and 1 component was sufficient to explain 81% of total variance.

A multinomial logistic regression model was built with the serum-based status of hypothyroid, euthyroid, and hyperthyroid as the outcome; principal component of markers, age, and sex assigned at birth (hereinafter: sex) were continuous predictors. The euthyroid group was used as the reference. The model was validated with bootstrapping of 1000 samples. Model performance metrics were determined based on confusion matrices and receiving operator characteristic curves (19). A predictive model was deployed on the AMIO sample, and the confusion matrices of CALIB and AMIO predictions were analyzed with 2 × 2 contingency tables. The tables compare the coincidence of serum- and HF-based prediction into hypothyroid, euthyroid, or hyperthyroid categories with and without amiodarone. Prediction was considered correct if the HF-based prediction coincided with the serum-based category of subject. Fisher's corrected χ^2 test with 95% confidence was used to assess the statistical significance of the interdependence of variables. Effect sizes are given as odds ratios, and all CI are of 95%.

Animal Studies

Animals and treatments

We used the THAI transgenic mouse model for our animal studies (18). In short, all tissues of the mouse express a *Luciferase* reporter gene under the control of a minimal viral promoter and a triplicate of the TH response element of the human *Dio1* promoter, which allows quantitative assessment of TH action in tissues by eliminating the influence of other response elements harbored by endogenous genes.

Studies were performed in a split-plot design with 1-way treatment structure for both experimental units; outlined in Fig. 1C. Male, 2- to 3-month old THAI mice were used and 2 to 4 animals were housed in each cage. Cages were randomly allocated into treated and control groups. Mice in treated cages received a chow diet containing amiodarone hydrochloride at a concentration of 1.35 g/kg (custom order from SSniff Specialdiäten GmbH; amiodarone hydrochloride obtained from Merck) for 8 weeks (20), while control animals received normal chow diet. On the last 2 days of the experiment, animals in each cage were randomly allocated into the vehicle or T4 group and received vehicle or 166.7 ng/bwg/day T4 intraperitoneally (n = 12-13/group) (18). As in our initial study T4 treatment did not result in a significant increase of cardiac *Luciferase* mRNA in mice not receiving amiodarone, likely due to the high TH degrading capacity of this tissue (21); in the experiment to investigate the heart we used ng/bwg/day T4 treatment instead of 166.7 ng/bwg/day T4 (n = 9-10/group). One day after the last T4 treatment, the animals were sacrificed. Blood was collected and tissue samples were dissected. Tissues were flash frozen on powdered dry ice and stored at −80 °C.

fT4 measurement in THAI mice

Serum fT4 was measured with AccuLite CLIA microwells kit (Monobind Inc.) according to the manufacturer's instructions in a Luminoskan Ascent (Thermo Fisher Scientific) machine.

Sample preparation and Taqman qPCR

Analysis of *Luciferase* gene expression was performed as described earlier (22). Total RNA was isolated from lung and brown adipose (BAT) tissues with the NucleoSpin RNA kit (Macherey-Nagel) according to the manufacturer's instructions. High-Capacity cDNA reverse transcription kit (Thermo Fisher Scientific) was used to reverse transcribe 1 µg of undiluted total RNA isolate, after which cDNA concentration of products was measured with the Qubit ssDNA assay kit (Invitrogen). All Taqman reactions were assayed with 50 ng of cDNA on a Viia7 instrument (Applied Biosystems). *Hprt1* showed low variability

Table 5. Bootstrap validation of prediction model

		Estimate	Bias	SE	95% CI		*P*
					Lower	Upper	
Predicting hyperthyroidism	Intercept	−0.256	−0.019	0.967	−2.22	1.72	.75
	Marker genes	−0.727	−0.053	0.179	−1.18	−0.48	.001
	Sex	0.256	−0.018	0.528	−0.78	1.31	.61
	Age	−0.012	0.000	0.019	−0.05	0.02	.47
Predicting hypothyroidism	Intercept	−1.283	−0.068	0.776	−3.02	0.06	.065
	Marker genes	0.491	0.027	0.230	0.12	0.98	.015
	Sex	0.869	0.053	0.524	−0.11	2.01	.074
	Age	0.001	0.000	0.015	−0.03	0.03	.94

Bootstrap validation of prediction model for classifying subjects into hypothyroid, euthyroid, or hyperthyroid based on novel tissue markers. Table shows results of 1000 samples. The euthyroid group served as reference group.

Table 6. Performance metrics of the prediction model

	Predict hypothyroidism	Predict euthyroidism	Predict hyperthyroidism
Precision	0.86	0.70	0.86
Sensitivity	0.79	0.80	0.81
Specificity	0.83	0.64	0.82
Negative predictive value	0.74	0.75	0.76
Area under curve	0.76	0.66	0.84
Total accuracy of the model	0.57		

Performance measures of prediction model for classifying subjects into hypothyroid, euthyroid, or hyperthyroid based on novel tissue markers.

and no significant difference between treatment groups and was used as the reference gene. The Taqman gene expressions assays are listed in Table 1 (Thermo Fisher Scientific).

Statistical analysis for animal studies

Data were organized with Microsoft Excel. Statistical analysis was done with Tibco STATISTICA v14. GraphPad Prism 9 and Adobe Illustrator were used to create figures. Data was analyzed with 2-way crossed analysis of variance followed by the Tukey post hoc test with 95% confidence level. Models were deemed adequate based on residual plots and normal residual plots. In order to make figures more comprehensible, gene expression data were normalized to the mean of vehicle on control diet group and multiplied by minus 1 (–ddCt). One unit increase in –ddCt means a 2-fold increase in gene expression.

Results

Human Study

Characteristics of subjects

A total of 315 participants were enrolled from May 2016 to August 2023; 194 were CALIB and 121 AMIO participants. Final sample size was determined after exclusion criteria had been applied. Baseline characteristics of eligible subjects can be found in Table 2.

Development of a hair follicle–based approach

For assessing human tissue TH signaling, next-generation sequencing of HF samples from subjects with hypothyroidism, euthyroidism, and hyperthyroidism (n = 5/group) revealed 1633 differentially expressed transcripts as potential biomarker candidates of tissue TH action (Fig. 2).

The TH regulation of 17 selected genes was validated with qPCR on a subset of CALIB samples (n = 45). Analysis of covariance was used to determine estimated means and CI of hypothyroid, euthyroid, and hyperthyroid groups. Four genes, bone morphogenic protein 2 (*Bmp2*), disheveled associated activator of morphogenesis 2 (*Daam2*), phospholipase A and acyltransferase 1 (*Plaat1*), and serine protease 27 (*Prsss27*) showed adequate basal expression in euthyroid samples and 2.7- to 6.5-fold difference in gene expression between hypothyroid and hyperthyroid cases in this subsample, and were selected as tissue TH markers for further investigation (Fig. 3).

Expression of selected marker genes was measured by Taqman qPCR from CALIB samples (n = 146) and were used as predictors in a logistic regression model. The model was designed to categorize tissue TH status as hypothyroid, euthyroid, or hyperthyroid, in accordance with serum-based TH status (Tables 3 and 4). The model was validated by bootstrapping and showed good performance characteristics (Tables 5 and 6 and Fig. 4). This shows that in the CALIB sample, serum-based TH status is reflected at the tissue level, and the 4 selected biomarker genes allow the assessment of tissue TH action. Consequently, the prediction model results allow investigating cases where serum-based classification might be inadequate to assess TH economy.

Amiodarone decreases the coincidence of serum-based and HF-based tissue TH status in patients with hyperthyroidism

Next, we deployed our prediction model to predict tissue TH status of AMIO patients (Table 7). To investigate if amiodarone impacts the concordance of serum- and HF-based classifications, the confusion matrices of the 2 samples were combined into 2 × 2 contingency tables where 1 variable is amiodarone treatment, and the other is if serum-based and HF-based TH statuses coincide (ie, if serum-based status is reflected at the tissue level) (Table 8 and Fig. 5).

In hypothyroid and euthyroid cases, amiodarone treatment had no significant effect on the coincidence of serum-based and HF-based prediction of TH status. However, in AMIO patients with hyperthyroidism, the coincidence of serum-based and HF-based categories of TH status was markedly lower. We found that there is only a 0.31 times chance that serum-based hyperthyroid status is concordant with HF-based TH status of AMIO patients compared with hyperthyroid CALIB patients.

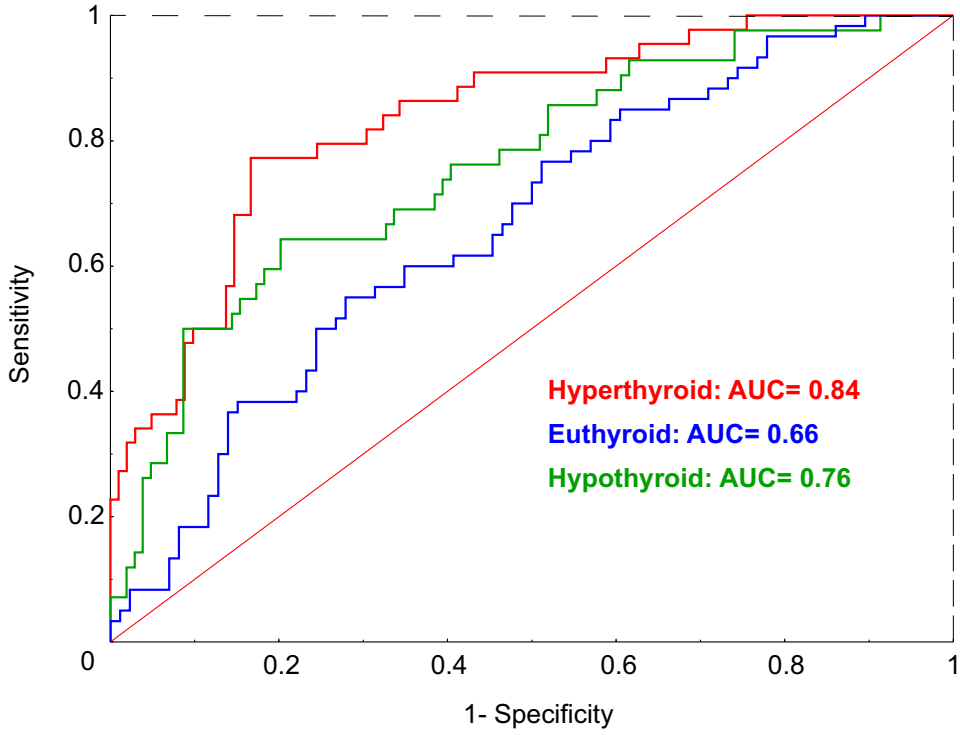

Figure 4. Receiving operator characteristic (ROC) curves of prediction model. ROC curves of prediction model for classifying subjects into hypothyroid, euthyroid, or hyperthyroid based on novel tissue markers. Abbreviation: AUC, area under curve.

Table 7. Confusion matrix of prediction model on AMIO sample

	Predicted hypothyroid	Predicted euthyroid	Predicted hyperthyroid
Observed hypothyroid	6	14	1
Observed euthyroid	9	34	2
Observed hyperthyroid	2	14	6

Confusion matrix of AMIO sample after deploying prediction model for classifying patients into hypothyroid, euthyroid, or hyperthyroid based on novel tissue markers. Observed means serum-based, predicted means tissue marker-based categorization.
Abbreviations: AMIO, amiodarone-treated individual.

In our study, 73% of AMIO patients with serum-based hyperthyroidism were not hyperthyroid at the tissue level, indicating that serum biochemistry overestimates the biological consequences of AIH, and tissue hyperthyroidism is present only in a smaller subgroup of patients. Our findings suggest that amiodarone treatment attenuates tissue TH action under hyperthyroid conditions, and patients with AIH are less likely to manifest tissue hyperthyroidism than other patients with hyperthyroidism.

Animal Studies

Amiodarone mitigates local TH action in hyperthyroid THAI mice

The interaction of amiodarone treatment and hyperthyroidism was further studied in THAI mice to validate human findings and to investigate whether this effect of amiodarone is also exerted in other tissues that are difficult to sample in humans. Mice received either normal or amiodarone-containing chow for 8 weeks (20). Half of the mice in each diet group received vehicle injection, while the other half were made hyperthyroid by T4 injections on the last 2 days of the experiment (Fig. 1A).

T4 treatment greatly increased serum fT4 level in animals on either diet, indicating serum-based hyperthyroidism (Fig. 6A and 6D). In animals on a control diet, the increased fT4 level led to increased *Luciferase* expression, the marker of TH signaling in THAI mice (18) in the lungs, a tissue known to be impacted by amiodarone (23), in the highly TH-responsive BAT (24) and importantly also in the heart, which serves as the primary target of amiodarone and is also greatly impacted by TH (25, 26) (Fig. 6B, 6C, and 6E). Under euthyroid conditions, amiodarone treatment had no effect on local TH action in any studied tissue. However, importantly, amiodarone prevented T4-induced increase of TH signaling in all tissues investigated (Fig. 6B, 6C, and 6E).

These findings indicate that amiodarone inhibits the tissue effects of TH under hyperthyroid conditions by mitigating the development and severity of tissue hyperthyroidism; this may be critically important when assessing risk of interventions in certain clinical scenarios in individual patients. Additionally, this occurs in multiple tissues, suggesting a global, non-tissue–specific phenomenon.

Discussion

Routine diagnosis of TH status has been greatly relying on the measurement of circulating TSH levels in the past 50 years. TSH-based treatment decisions became highly useful in improving the condition of millions of patients. However, during the last decades, it has become clear that besides the tight regulation of circulating TH levels by the hypothalamo–pituitary–thyroid axis, tissue-specific cellular regulatory mechanisms also influence TH action (2, 27). This machinery ensures that local TH action can be highly different in certain tissues despite

Table 8. Contingency tables and statistics of human data

	Prediction correct	Prediction incorrect
Hypothyroid		
AMIO	6	15
CALIB	20	22
P	.18	
OR	0.44	
95% CI	0.14-1.35	
Euthyroid		
AMIO	34	11
CALIB	39	21
P	>.99	
OR	1.66	
95% CI	0.70-3.94	
Hyperthyroid		
AMIO	6	16
CALIB	24	20
P	.041	
OR	0.31	
95% CI	0.10-0.95	
All samples		
AMIO	46	42
CALIB	83	63
P	.50	
OR	0.83	
95% CI	0.49-1.41	

Contingency tables of comparing AMIO and CALIB samples. Tables were analyzed with Fisher's corrected χ^2 test with 95% CI. Prediction was considered correct if HF-based prediction coincided with serum-based category of subject. Abbreviations: AMIO, amiodarone-treated individual; CALIB, not amiodarone–treated calibrator.

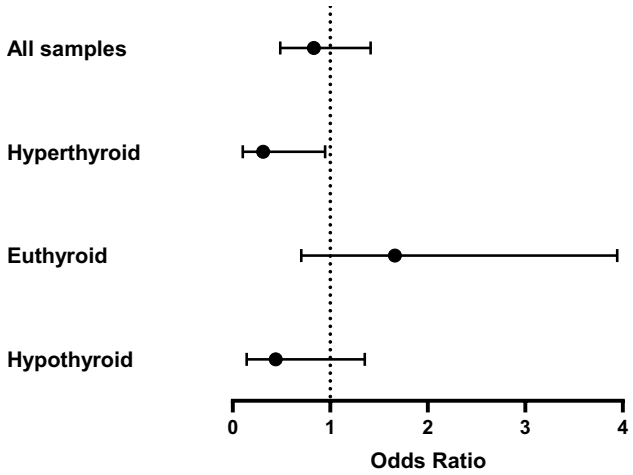

Figure 5. Forest plot of odds ratios from contingency tables. Visual representation of odds ratios with 95% CI from contingency tables of Table 8.

stable circulating TH levels. Furthermore, under specific pathophysiological conditions, misregulation of the local regulatory machinery can uncouple circulating TSH and TH levels from tissue TH actions (eg, in RTHα syndrome when normal circulating TSH and TH levels are accompanied by severe tissue hypothyroidism in TRα dominant tissues) (28); importantly, considering serum TH levels hardly resolve these contradictions (2). Although a large patient group would benefit from assessing TH action directly from tissues, such attempts are greatly challenging (29, 30).

Several biomarkers of tissue TH action in humans have already been described, although their clinical relevance is yet to be demonstrated (29). Sampling of patients should be conducted in a minimally invasive way, which is a considerable limitation in identifying new tissue markers. Since blood is regularly drawn from patients and subjected to serum biochemistry, it would be convenient to find markers of tissue TH action in the blood. In fact, several circulating markers have already been proposed, although many of the data are inconclusive (29, 31). Nock et al reported the circulating peptide CD5L, originating from the liver, spleen, and bone tissues, to be a potentially good biomarker of tissue TH action, which was also confirmed in animal and cellular models (32). However, the level of a secreted molecule is the net result of various biological processes that occur after TH exerts its effects on tissues and could be considered a more indirect marker, which raises concerns about its representativeness.

The blood also consists of cellular elements like white blood cells, and the immune system is known to be strongly regulated by TH action (33). Massolt et al reported TH-sensitive white blood cell transcriptome and proposed leukocyte-originated markers as measures of tissue TH action in TRα dominant tissues (34). In this study, the obtained transcriptome was also compared with the skeletal muscle transcriptome they recorded earlier (35). They concluded some of the proposed marker genes to be representative of skeletal muscle TH action, arguing for general representativeness of leukocyte markers.

Due to easy sampling and well-characterized TH sensitivity (36), we tested whether an HF transcriptome–based approach can accurately measure tissue TH status of patients. In contrast to blood-originated markers, HFs are tissue-embedded cellular systems with more complex biological barriers than white blood cells and therefore can be considered more representative for tissues less exposed to changes in serum TH levels and express hundreds of TH-responsive genes. While the transcriptome analysis revealed that among the TH-responsive genes in this tissue many are regulated only either by hypothyroidism or hyperthyroidism, several genes were found to be regulated by both conditions, making them suitable biomarker candidates for assessing tissue TH status. Based on their easily detectable basal expression in euthyroidism and the high influence of TH status on their expression level, 4 genes were selected as biomarkers: *Bmp2*, *Daam2*, *Plaat1*, and *Prss27*. The expression level of these genes in the HFs correlated with serum-based TH status of CALIB subjects.

We aimed to develop a clinical tool to estimate tissue TH status of individual subjects. However, this requires the assessment of predictive power. Therefore, we created a classification model to categorize subjects as hypothyroid, euthyroid, or hyperthyroid at the tissue level using the data gathered from the CALIB sample. The combined use of the 4 marker genes instead of 1 has the advantage of making the model biologically more robust by mitigating biases caused by biological events unrelated to TH status. We validated this model with bootstrapping, and the performance metrics showed high values. Thus, based on their HF gene expression pattern, the generated classification model is fit to categorize subjects into

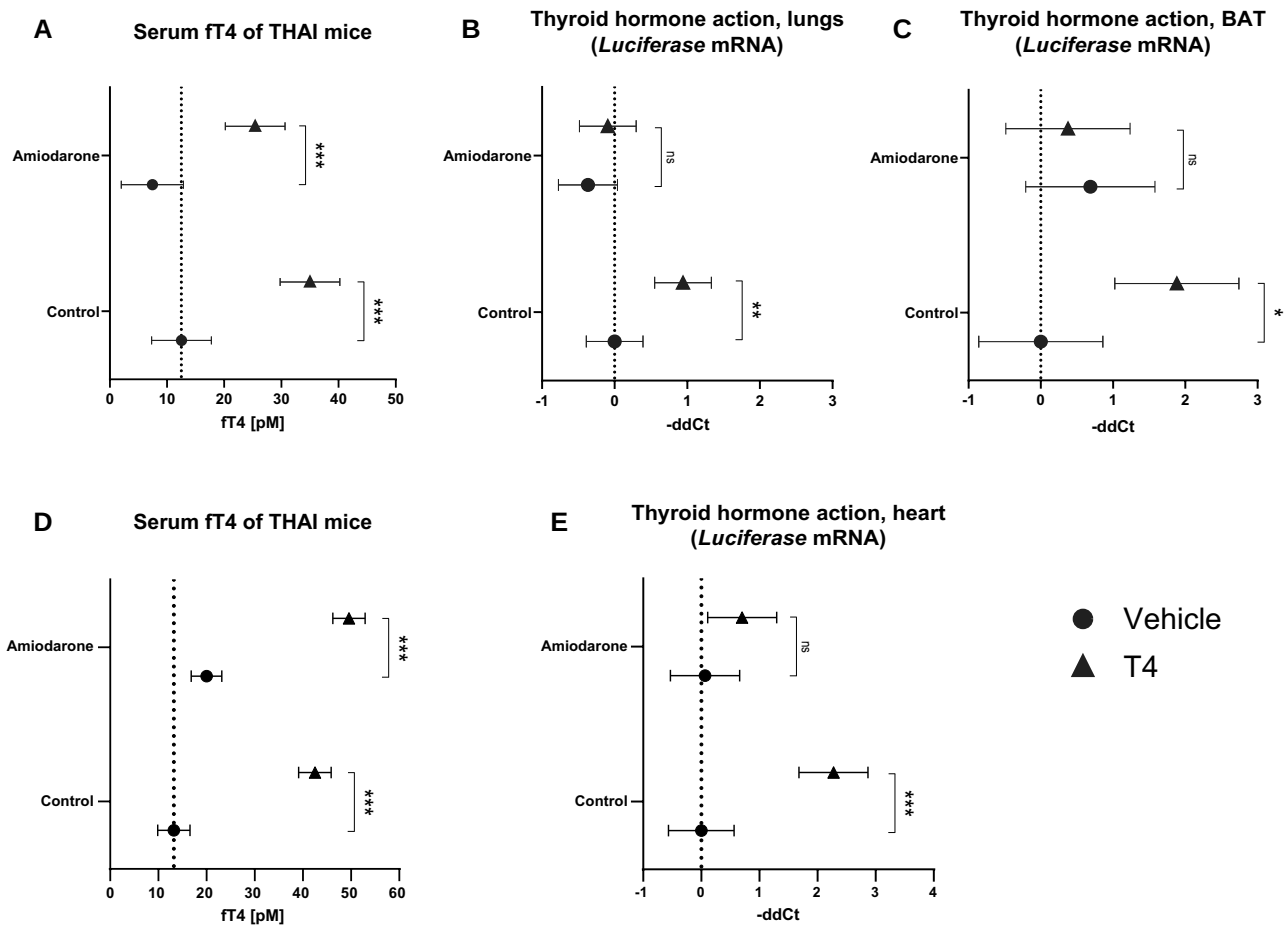

Figure 6. Serum fT4 levels and local thyroid hormone action in lungs, brown adipose tissue, and heart of THAI mice after amiodarone and T4 treatment. Male THAI mice were treated with amiodarone hydrochloride in the diet (1.35 mg/g chow) for 8 weeks and received 166.7 ng/bwg/day (A-C) or 250 ng/bwg/day (D, E) T4 intraperitoneally on the last 2 days. Tissue thyroid hormone action is measured by *Luciferase* mRNA. One unit increase in –ddCt means a 2-fold increase in gene expression. The figure shows estimates of means ± 95% CI from 2-way crossed analysis of variance model. (A) serum fT4 levels (T4: 166.7 ng/bwg/day). (B) Tissue thyroid hormone action in the lungs. (C) Tissue thyroid hormone action in the BAT. (D) Serum fT4 levels (T4: 250 ng/bwg/day). (E) Tissue thyroid hormone action in the heart. n = 12-13/group (A-C) or 9-10/group (D, E). Abbreviations: T4, thyroxine; BAT, brown adipose tissue; mRNA, messenger RNA; $*P < .05$; $**P < .01$; $***P < .001$.

tissue hypothyroid, euthyroid, and hyperthyroid categories. The current approach targeting tissue TH economy may be translated into personalized medicine, especially in untypical cases where tissue TH action is not directly related to serum biochemistry.

Amiodarone is widely used for the treatment of non–life-threatening supraventricular and for the treatment and prevention of life-threatening ventricular arrhythmias (5, 37). In up to 10% and 1% of patients, amiodarone treatment leads to hypothyroidism and AIH, respectively, as defined by the respective changes in serum TSH and TH levels (5). While amiodarone-induced hypothyroidism can be treated by T4 supplementation, and the patient may remain on amiodarone, according to the current guidelines (6, 7), the diagnosis of AIH requires the termination of amiodarone treatment. If, for any reason, cessation of the drug is not possible (ie, amiodarone is the only effective medication for a returning life-threatening arrhythmia), thyroidectomy is required.

Results of in vitro and in vivo rodent experiments raised the possibility that in addition to its effect on thyroidal TH production and consequent alteration of serum levels, amiodarone may also influence the local machinery of TH metabolism and signaling in tissues (5, 9, 11, 12, 17). In addition, many patients with AIH exhibit only mild hyperthyroidism–related symptoms or no symptoms at all, despite their unusually high serum TH levels. This discrepancy raises the possibility that amiodarone markedly attenuates tissue TH action (5-7).

We deployed our classification model to test whether serum-based diagnosis of AIH is reflected in the gene expression pattern of HFs. We found only a 0.31 times the chance for correspondence between serum-based and HF-based classifications of TH status in patients with AIH compared with patients with non-amiodarone–induced hyperthyroidism. In accordance with this, in 73% of studied patients with AIH, serum biochemistry was not reflected in the tissues, indicating that amiodarone can modulate the cellular effects of high circulating TH levels. Importantly, this argues for assessing tissue TH action in patients with AIH to support treatment decisions and determine whether amiodarone could be continued (eg, before cardiac intervention or heart transplantation). To further evaluate the effects of amiodarone on tissue TH action under hyperthyroid conditions, we studied the interaction of amiodarone and high T4 level on tissue TH action in THAI mice. In animals on an amiodarone-free diet, T4 treatment induced marked elevation of TH action both in

the lungs, an organ greatly impacted by amiodarone (23) and in the TH-sensitive BAT (24) and in the heart, a major target of both molecules (25, 26). However, amiodarone treatment abolished the T4-induced elevation of TH action in all investigated tissues despite the highly increased serum fT4. These data show that long-term amiodarone treatment in humans and mice markedly attenuates tissue TH action in multiple tissues, but only when circulating TH levels are elevated. More studies are necessary to understand the mechanism of this phenomenon.

The notion that cardiac TH action is protected in amiodarone-treated patients may overestimate the risks of hyperthyroid serum biochemistry. This proposes the re-evaluation of current risk assessment of TH related factors in these cases and emphasizes the need of a method to provide better estimates on tissue TH status in humans.

We note that our study is based on a specific geographical region with relatively homogeneous iodine availability that is known to affect amiodarone-induced thyroid dysfunction. Despite this limitation, 1 of the strengths is that we confirmed our human findings in multiple tissues of mice. This highlights that our human HF-based approach could be used as a general assessment of tissue TH action in peripheral tissues, and could be used to study other untypical patient cases. This would be of considerable importance in conditions where monitoring treatment efficiency is challenging, like in RTHβ and especially RTHα syndromes, or during T4 supplementation if the patient has persisting symptoms of hypothyroidism despite having TSH in the normal range.

In conclusion, we developed a novel, minimally invasive approach to measure tissue TH action in humans and built a classification model to help evaluate tissue TH action of individual subjects. The classification model has been validated and showed good predictive performance and may serve as a complementary tool for clinical scenarios when unexplained discrepancies between routinely measured serum parameters and clinical symptoms are present. Furthermore, we found that amiodarone manifested the dissociation of serum biochemistry and tissue TH action; however, this was seen only in patients with AIH, while it was missing in individuals with euthyroidism and individuals with hypothyroidism treated with amiodarone. Results from our THAI model further confirmed amiodarone-induced attenuation of tissue TH action under hyperthyroid conditions and support the representative nature of our human biomarker system. These concordant studies suggest that TSH and fTH levels may be insufficient to assess TH status of amiodarone-treated patients, strongly arguing for estimating their tissue TH economy before clinical decision-making.

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Author Contributions

Every author had full access to all data and accept responsibility to submit for publication. All authors provided contribution statement in the requested form. Conceptualization: B.G. Cs.F., E.V.N., P.C., R.S. Data Curation: M.K., R.S., B.D., D.K. Formal analysis: R.S., P.C. Funding acquisition: B.G., Cs.F. Investigation: R.S., D.K., B.D., M.K., O.D., G.L.K., G.T., T.F. Methodology: R.S., P.C., B.G., Cs.F., E.V.N. Project administration: B.G., Cs.F., E.V.N., M.K., R.S. Resources: E.V.N., O.D., G.L.K., T.F., G.T., M.K., B.G., Cs.F., P.C. Supervision: B.G., Cs.F., E.V.N. Validation: R.S., M.K., P.C., B.G., Cs.F., E.V.N. Visualization: R.S., B.D., D.K., B.G., Cs.F. Writing—original draft: R.S., B.G., Cs.F., E.V.N., M.K., O.D. Writing—review and editing: R.S., B.G., Cs.F., E.V.N., M.K., G.L.K., O.D., T.F., P.C., G.T.

Disclosures

R.S. received a travel grant from the European Thyroid Association to present parts of this work at the 45th Annual Meeting of the European Thyroid Association. No other author has anything to disclose.

Data Availability

Some or all datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request. The corresponding authors had full access to all data and final responsibility for the decision to submit for publication.

Ethics Approval

The human study was approved by the National Center for Public Health and Pharmacy of Hungary (OGYÉI/54197-2/2023). All participants provided written and informed consent. Animal studies were approved by the Animal Welfare Committee at the HUN-REN Institute of Experimental Medicine, Hungary (PE/EA/1490-7/2017).

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