Method for determining tissue thyroid status in humans

FIELD OF THE INVENTION

The invention relates to the field of determining the tissue thyroid status of a subject. A minimally-invasive approach using hair follicle markers to assess tissue thyroid hormone (TH) economy is provided. A classification model fit to categorize subjects into tissue hypothyroid, euthyroid and hyperthyroid categories is provided. The method is suitable to determine tissue thyroid status in patients treated with amiodarone and diagnosed as hyperthyroid or hypothyroid and exhibiting mild or no symptoms of hyperthyroidism or hypothyroidism, respectively.

BACKGROUND OF THE INVENTION

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 to improve 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 (HPT) axis, tissue-specific cellular regulatory mechanisms also influence TH action (Gereben B, McAninch EA, Ribeiro MO, Bianco AC. Scope and limitations of iodothyronine deiodinases in hypothyroidism. Nat Rev Endocrinol. 2015;11(11):642-52.). This machinery ensures that local TH action can be highly different in certain tissues despite of 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, e.g. in TH resistance alpha syndrome when normal circulating TSH and TH levels are accompanied with severe tissue hypothyroidism in TH receptor alpha dominant tissues (Gereben B, Zavacki AM, Ribich S, Kim BW, Huang SA, Simonides WS, et al. Cellular and molecular basis of deiodinase-regulated thyroid hormone signaling. Endocr Rev. 2008;29(7):898-938.). Importantly, consideration of serum TH levels hardly resolve these contradictions. In situations when the clinical symptoms and serum biochemistry are equivocal, mere TSH- and free TH- (fTH) based approaches are questionable. Although a large patient group would benefit from assessing TH action directly from tissues, such attempts are greatly challenging (Jansen HI, Bruinstroop E, Heijboer AC, Boelen A. Biomarkers indicating tissue thyroid hormone status: ready to be implemented yet? J Endocrinol. 2022;253(2):R21-R45.).

Several biomarkers of tissue TH action in humans have already been described, which include but are not exclusive to TH sensitive marker genes used in basic research (e.g. Hönes et al. Canonical Thyroid Hormone Receptor β Action Stimulates Hepatocyte Proliferation in Male

Mice, Endocrinology, Volume 163, Issue 3, March 2022, bqac003 indicating Ccnd1; Zambrano et al. The thyroid hormone receptor β induces DNA damage and premature senescence. J Cell Biol (2014) 204 (1): 129–146. indicating Co1; Dio1 and Dio3 in e.g. Gereben Activation and inactivation of thyroid hormone by deiodinases: Local action with general consequences. Cell. Mol. Life Sci. 65, 570–590 (2008); Enpp2 in Haas et al. Microarray analysis of thyroid hormone-induced changes in mRNA expression in the adult rat brain. Neuroscience Letters, Volume 365, Issue 1, 2004, Pages 14-18), although their clinical relevance is yet to be demonstrated. Most known biomarkers are blood-related, either secreted or otherwise present in the blood, or white blood cell originated (Massolt ET, Meima ME, Swagemakers SMA, Leeuwenburgh S, van den Hout-van Vroonhoven M, Brigante G, et al. Thyroid State Regulates Gene Expression in Human Whole Blood. J Clin Endocrinol Metab. 2018;103(1):169-78.) Despite of the advantage of easy sampling, the usefulness of secreted circulating markers is limited as their synthesis, secretion and metabolism are under complex regulation that is influenced by a series of non TH-related factors of multiple organs (Jansen HI, Bruinstroop E, Heijboer AC, Boelen A. Biomarkers indicating tissue thyroid hormone status: ready to be implemented yet? J Endocrinol. 2022;253(2):R21-R45.). The potential failure of circulating TSH and TH levels to predict tissue TH action in specific 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.

A patient group in which such controversies are common are patients on amiodarone treatment. Amiodarone is an antiarrhythmic drug with a potential to alter serum TH levels leading to either hypo- or hyperthyroidism (Trohman RG, Sharma PS, McAninch EA, Bianco AC. Amiodarone and thyroid physiology, pathophysiology, diagnosis and management. Trends Cardiovasc Med. 2019;29(5):285-95.). 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 (Trohman RG, Sharma PS, McAninch EA, Bianco AC. Amiodarone and thyroid physiology, pathophysiology, diagnosis and management. Trends Cardiovasc Med. 2019;29(5):285-95.). This discrepancy has been studied before, but the underlying mechanisms are still vaguely known. 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. While amiodarone induced hypothyroidism can be treated by T4 supplementation and the patient may remain on amiodarone, according to the current guidelines (De Leo S, Lee SY, Braverman LE. Hyperthyroidism. Lancet. 2016;388(10047):906-18.), the diagnosis of AIH

requires the termination of amiodarone treatment. If for any reason cessation of the drug is not possible, i.e. amiodarone is the only effective medication for a returning life-threatening arrhythmia, thyroidectomy is required.There is thus a need for a clinical tool to estimate tissue TH status of individual patients.

SUMMARY OF THE INVENTION

A novel, minimally invasive approach to measure tissue TH action in humans, and a classification model to evaluate tissue TH action of individual patients are provided.

In a first aspect a method for determining tissue thyroid status of a subject (i.e. whether the subject is euthyroid, hypothyroid or hyperthyroid at the tissue (i.e. not systemic, blood or serum) level) is provided, the method comprising

i) measuring the level of expression of at least one biomarker selected from the group consisting of Bmp2, Daam2, Plaat1 and Prss27 or at least one biomarker selected from the group consisting of Bmp2, Daam2, Plaat1, Prss27, Wfdc12 and Yjefn3, in a hair follicle sample of the subject,

ii) comparing the level of expression measured in i) to a corresponding reference value,

iii) determining tissue thyroid status of a subject based on the comparison of ii).

In another aspect a method of treating a subject suffering from tissue hyperthyroidism or tissue hypothyroidism is provided, the method comprising

i) measuring the level of expression of at least one biomarker selected from the group consisting of Bmp2, Daam2, Plaat1 and Prss27 or at least one biomarker selected from the group consisting of Bmp2, Daam2, Plaat1, Prss27, Wfdc12 and Yjefn3, in a hair follicle sample of the subject,

ii) comparing the level of expression measured in i) to a corresponding reference value,

iii) determining whether the subject has tissue hyperthyroidism or tissue hypothyroidism based on the comparison and

iv) treating the subject by dietary and/or pharmaceutical intervention to restore tissue euthyroid status.

In another aspect the use of a biomarker selected from the group consisting of Bmp2, Daam2, Plaat1 and Prss27 or a biomarker selected from the group consisting of Bmp2, Daam2, Plaat1, Prss27, Wfdc12 and Yjefn3, for determining the tissue thyroid status of a subject is provided.

Preferably the use comprises

i) measuring the level of expression of at least one biomarker selected from the group consisting of Bmp2, Daam2, Plaat1 and Prss27 or at least one biomarker selected from the group

consisting of Bmp2, Daam2, Plaat1, Prss27, Wfdc12 and Yjefn3, in a hair follicle sample of the subject,

ii) comparing the level of expression measured in i) to a corresponding reference value

iii) determining tissue thyroid status of a subject based on the comparison of ii).

The following embodiments are preferred embodiments of any of the aspects of the invention.

Preferably comparing the level of expression of the at least one biomarker comprises

a) calculating one or more indices characteristic of the subject based on the measured expression level and

b) comparing the one or more indices calculated in a) to one or more indices calculated from the level of expression of the at least one biomarker and optionally the amount of the additional biomarker measured in a reference sample.

Preferably the reference sample comprises at least one, preferably a plurality of hyperthyroid samples or at least one, preferably a plurality of hypothyroid samples or at least one, preferably a plurality of euthyroid samples or a combination thereof.

Preferably the reference sample comprises or consists of at least one, preferably a plurality of hyperthyroid samples, at least one, preferably a plurality of hypothyroid samples and at least one, preferably a plurality of euthyroid samples.

Preferably calculating the one or more indices characteristic of the subject based on the measured expression level(s) comprises conducting a principal component analysis on the (expression levels of the) biomarkers and optionally using other variables (such as the age or sex of the subject; parameter characteristic of the subject).

Preferably calculating the one or more indices calculated from the level of expression of the at least one biomarker measured in a reference sample comprises conducting a principal component analysis on the markers and optionally using other variables (such as the age or sex of the subject; parameter characteristic of the subject);

building a multinomial logistic regression model with serum thyroid function test based status of hypothyroid, euthyroid and hyperthyroid as outcome, using the one or more, preferably a plurality of euthyroid samples as reference.

Preferably the at least one biomarker selected from the group consisting of Bmp2, Daam2, Plaat1 and Prss27 or from the group consisting of Bmp2, Daam2, Plaat1, Prss27, Wfdc12 and Yjefn3, is Prss27.

Preferably the at least one biomarker selected from the group consisting of Bmp2, Daam2, Plaat1 and Prss27 or from the group consisting of Bmp2, Daam2, Plaat1, Prss27, Wfdc12 and Yjefn3, are Bmp2, Daam2, Plaat1 and Prss27.

Preferably the at least one biomarker selected from the group consisting of Bmp2, Daam2, Plaat1, Prss27, Wfdc12 and Yjefn3 are Bmp2, Daam2, Plaat1, Prss27, Wfdc12.

Preferably the at least one biomarker selected from the group consisting of Bmp2, Daam2, Plaat1, Prss27, Wfdc12 and Yjefn3 are Bmp2, Daam2, Plaat1, Prss27, Wfdc12 and Yjefn3.

Preferably the at least one biomarker is Wfdc12.

Preferably the at least one biomarker is Yjefn3.

Preferably the subject receives or have received a substance affecting tissue thyroid status. Preferably the substance affects tissue thyroid status and serum TSH, free T4, or T3 levels differently.

Preferably the subject is treated or has been treated with amiodarone.

Preferably the subject is diagnosed as hyperthyroid or hypothyroid when using a serum thyroid function test.

Preferably the subject is diagnosed as hyperthyroid or hypothyroid using a serum thyroid function test and exhibits mild or no symptoms of hyperthyroidism or hypothyroidism, respectively.

Preferably the subject receives a substance inducing hyperthyroidism or hypothyroidism.

Preferably the substance is amiodarone. Preferably the substance is lithium, a checkpoint inhibitor, alemtuzumab, interferon-alfa, thyroxine or iodine.

Preferably the subject has a condition inducing hyperthyroidism or hypothyroidism.

Preferably the expression level(s) is determinded in vitro.

Preferably the subject is a human.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Biomarker candidates for tissue TH action in human hair follicles of the CALIB sample. Figure shows the expression levels of marker genes as mean ± 95 % CI estimated with analysis of covariance. One unit increase in –ddCt means twofold increase in gene expression. Euthyroid group was reference group. N(accepted markers)=148, n(rejected markers)= 45; *Prss27*: serine protease 27; *Daam2*: dishevelled 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.

Figure 2. Receiving operator characteristic (ROC) curves of the 4 gene prediction model for classifying patients into hypo-, eu-, or hyperthyroid based on novel tissue markers. A: ROC curve of hyperthroid class; B: ROC curve of euthroid class; C: ROC curve of hypothyroid class.

Figure 3. Forest plot of odds ratios from contingency tables. Visual representation of OR from contingency tables of Table 10.

Figure 4. 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.

Figure 5. Flowchart of the human study.

Figure 6. Expression of WAP Four-Disulfide Core Domain 12 mRNA (*Wfdc12*) in hypothyroid, euthyroid and hyperthyroid patient' hair follicles. Parameters estimated by analysis of covariance; n= 105; dCt: threshold cycle normalized to reference (one unit increase in dCt means two-fold increase in expression); *: $p< 0.05$.

Figure 7. Expression of YjeF N-Terminal Domain Containing 3 (*Yjefn3*) in patient' hair follicles. Parameters estimated by analysis of covariance; n= 122; dCt: threshold cycle normalized to reference (one unit increase in dCt means two-fold increase in expression); **: $p< 0.01$.

DETAILED DESCRIPTION OF THE INVENTION

The terms "index", "indices" refer to an index (e.g. value) generated by a mathematical/statistical method from the measured expression level(s) (and optionally amount(s)) in a sample from a subject whose thyroid status is to be determined and optionally on other characteristics of the subject. A suitable mathematical/statistical method is described in the Examples. The terms "index" and "value" may be used interchangeably herein.

The term "serum thyroid function test" refers to tests assessing the function of the thyroid gland in serum samples. Serum thyroid function tests may assess (e.g. quantify) thyroid stimulating hormone (TSH), thyroxine (T4, such as total T4 and free T4), triiodothyronine (T3) etc.

"Determining tissue thyroid status" may be used interchangeably with determining whether a subject is hypo-, eu- or hyperthyroid at tissue level or at a certain tissue level. The tissue is preferably different from the blood (serum). "Tissue thyroid status" may be interpreted as the thyroid status in a (certain) tissue, that is, not a systemic thyroid status. Systemic thyroid status or "blood (serum) thyroid status" may be directly determined by serum based tests. The terms "thyroid status" and "thyroid function" may be used interchangeably herein. The term "tissue thyroid status of a subject" refers to the thyroid status (function) that is characteristic of a (certain) tissue, that is not the blood (serum). Both systemic and tissue thyroid status may be euthyroid, hypothyroid or hyperthyroid. A biomarker that is suitable to determine tissue thyroid status is one that is suitable to determine any of euthyroidism, hypothyroidism and hyperthyroidism. A biomarker that is suitable to determine tissue thyroid status is suitable to identify euthyroidism, hypothyroidism and hyperthyroidism at the tissue level. A biomarker that is suitable to determine tissue thyroid status is suitable for differentiating between tissue euthyroidism, hypothyroidism and hyperthyroidism. Accordingly, a biomarker that is suitable for identifying only one or two of euthyroidism, hypothyroidism and hyperthyroidism at the tissue level, may not be a biomarker that is suitable to determine tissue thyroid status as used herein.

Symptoms of hyperthyroidism may be e.g. weight loss despite an increased appetite, tachycardia, arrhythmia, heart palpitations, nervousness, anxiety, irritability, tremor, sleep problems, changes in menstrual cycles, fatigue, muscle weakness, sweating, frequent bowel movements, enlarged thyroid gland, lethargy, weakness, tiredness, increased sensitivity to heat, warm, moist skin, thinning skin, increased serum FT4 and FT3 levels, suppressed serum TSH level.

Symptoms of hypothyroidism may e.g. be fatigue, weight gain, trouble tolerating cold, joint and muscle pain, dry skin, heavy or irregular menstrual periods or fertility problems, bradycardia, depression, puffy face, hoarse voice, memory problems, constipation.

Routine diagnostics of thyroid function (status) are based on circulating central parameters, namely serum TSH and fTH levels. However, there is a large biological emphasis on the local regulatory system to render TH action tissue-specific. The discrepancy between clinical symptoms and TH status predicted by serum biochemistry, in certain disease conditions strongly argue for the development of tissue TH economy estimation tools, in order to improve clinical decision-making. Such cases include AIH, which may manifest with extremely high TH levels, while the accompanying clinical symptoms of hyperthyroidism are disproportionally mild. In order to resolve this discrepancy, it would be critical to determine the effect of TH at the level of target tissues of an individual, which has not been possible until now.

The herein provided minimally invasive qPCR-based approach uses easily accessible human tissue, hair follicle (HF) to assess tissue TH action. A transcriptome from HF of hypothyroid, euthyroid and hyperthyroid patients with Next Generation Sequencing was recorded and the expression of hundreds of TH sensitive genes in HF was demonstrated (Figure 1). As mentioned above, several biomarkers are strongly supported to be sensitive to thyroid hormones by the prior art, but it became clear from the transcriptome prepared from HFs of hypothyroid, euthyroid and hyperthyroid patients, that many of these were not suitable to use for diagnosis the invention relates to. For example, the expression of Dio1, Dio3 or Enpp2 was too low to use in a diagnostic method.

WAP Four-Disulfide Core Domain 12 (*Wfdc12)* and YjeF N-Terminal Domain Containing 3 (*Yjefn3*) were identified as thyroid hormone controlled genes and also as marker genes of hypothyroid, euthyroid and hyperthyroid states, which can be used in a diagnostic method based on HF. Besides, bone morphogenic protein 2 (*Bmp2),* disheveled associated activator of morphogenesis 2 *(Daam2),* phospholipase A and acyltransferase 1 *(Plaat1)* and serine protease 27 *(Prss27)* were also identified as predictors in a classification model to categorize patients into being hypothyroid, euthyroid or hyperthyroid at the tissue level.

Five of these markers, namely *Bmp2, Daam2, Plaat1, Wfdc12* and *Prss27* were included as predictors in a classification model to categorize patients into being hypothyroid, euthyroid or hyperthyroid at the tissue level. The combined use of the marker genes instead of one has the advantage of making the model biologically more robust by mitigating biases caused by biological events unrelated to TH status. The model was validated with bootstrapping. The generated classification model, herein provided, is fit to categorize patients into tissue hypothyroid, euthyroid and hyperthyroid categories based on their HF gene expression pattern.

The model comprising *Bmp2, Daam2, Plaat1* and *Prss27* and the 5-gene (*Bmp2, Daam2, Plaat1, Wfdc12* and *Prss27)* model were deployed to investigate tissue TH action in amiodarone treated patients. One of the reasons of choosing amiodarone treated patients was that many patients with AIH exhibit only mild hyperthyroidism-related symptomes or no symptomes at all, despite of their unusually high serum TH levels. The classification model was used to test whether serum-based diagnosis of AIH is reflected in the gene expression pattern of HFs. It was found that there was only a 0.31 times the chance for correspondence between serum-based and HF-based classifications of TH status in patients with AIH, compared to patients with non-amiodarone induced hyperthyroidism. In accordance with this, in 73% of studied AIH patients serum biochemistry was not reflected in the tissues. Data from this study show that AIH is not necessarily associated with tissue hyperthyroidism, a finding that is consonant with clinical

observations. This argues for assessing tissue TH action in patients with AIH to support treatment decisions and determine whether amiodarone could be continued, e.g. before cardiac intervention or heart transplantation. The diagnostic method herein provided is a useful tool to determine the tissue TH status of AIH patients.

Additional data from rodents show that in the Thyroid Hormone Action Indicator (THAI) mouse model amiodarone mitigates tissue TH action when serum T4 concentration is increased. 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. The method provided herein can serve as a (complementary) tool for clinical scenarios when unexplained discrepancies between routinely measured serum parameters and clinical symptomes are present. The data presented herein suggest that TSH and fTH levels may be insufficient to assess TH status of amiodarone treated patients, strongly arguing for estimation of their tissue TH economy before clinical decision-making.

The data presented herein support that the human HF-based markers provided herein are not only responsive to changes in serum hormone levels, but are also representative markers of tissue TH action of peripheral tissues.

In yet another aspect the method according to any one of the aspects comprises measuring the amount of an additional biomarker affected by tissue thyroid hormone action and comparing the amount of the additional biomarker to a corresponding reference value.

The following embodiments are preferred embodiments of any of the aspects of the invention.

Preferably the additional biomarker affected by tissue thyroid hormone action is a gene and the amount is the level of expression.

Preferably comparing the level of expression of the at least one biomarker and optionally the amount of additional biomarker to a corresponding reference value comprises

a) calculating one or more indices characteristic of the subject based on the measured expression level and optionally on the measured amount of the additional biomarker and

b) comparing the one or more indices calculated in a) to one or more indices calculated from the level of expression of the at least one biomarker and optionally the amount of the additional biomarker measured in a reference sample.

In all aspects the following are preferred embodiments.

Preferably an alteration of the expression level of the one or more biomarkers from the reference level of expression indicating euthyroidism indicates tissue hypothyroidism or tissue hyperthyroidism.

Preferably an elevated level of the one or more biomarkers compared to the reference level of expression indicating euthyroidism indicates tissue hyperthyroidism.

Preferably a decreased level of the one or more biomarkers compared to the reference level of expression indicating euthyroidism indicates tissue hypothyroidism.

Preferably an elevated level of the one or more biomarkers compared to the reference level of expression indicating hypothyroidism indicates tissue euthyroidism or tissue hyperthyroidism.

Preferably the at least one biomarker selected from the group consisting of Bmp2, Daam2, Plaat1 and Prss27 is Bmp2. Preferably the at least one biomarker selected from the group consisting of Bmp2, Daam2, Plaat1 and Prss27 is Daam2. Preferably the at least one biomarker selected from the group consisting of Bmp2, Daam2, Plaat1 and Prss27 is Plaat1.

Preferably the at least one biomarker selected from the group consisting of Bmp2, Daam2, Plaat1 and Prss27 are Prss27 and Daam2. Preferably the at least one biomarker selected from the group consisting of Bmp2, Daam2, Plaat1 and Prss27 are Prss27 and Bmp2. Preferably the at least one biomarker selected from the group consisting of Bmp2, Daam2, Plaat1 and Prss27 are Prss27 and Plaat1. Preferably the at least one biomarker selected from the group consisting of Bmp2, Daam2, Plaat1 and Prss27 are Daam2 and Bmp2. Preferably the at least one biomarker selected from the group consisting of Bmp2, Daam2, Plaat1 and Prss27 are Daam2 and Plaat1. Preferably the at least one biomarker selected from the group consisting of Bmp2, Daam2, Plaat1 and Prss27 are Bmp2 and Plaat1.

Preferably the at least one biomarker selected from the group consisting of Bmp2, Daam2, Plaat1 and Prss27 are Prss27, Daam2 and Bmp2. Preferably the at least one biomarker selected from the group consisting of Bmp2, Daam2, Plaat1 and Prss27 are Prss27, Daam2 and Plaat1. Preferably the at least one biomarker selected from the group consisting of Bmp2, Daam2, Plaat1 and Prss27 are Prss27, Bmp2 and Plaat1.

Highly preferably the at least one biomarker selected from the group consisting of Bmp2, Daam2, Plaat1 and Prss27 is Prss27. Highly preferably the at least one biomarker selected from the group consisting of Bmp2, Daam2, Plaat1 and Prss27 are Bmp2, Daam2, Plaat1 and Prss27.

Preferably an additional biomarker is selected from the group consisting of Wfdc12 and Yjefn3 in addition to the biomarker selected from the group consisting of Bmp2, Daam2, Plaat1 and Prss27. Preferably the additional biomarker is Wfdc12. Preferably the additional biomarker is Yjefn3. Preferably the additional biomarker is Wfdc12 and Yjefn3.

Preferably the additional biomarker is used in the method as the at least one biomarker selected from the group consisting of Bmp2, Daam2, Plaat1 and Prss27, i.e. its expression level is measured in a hair follicle sample of the subject and the measured expression level is used in the

method as the expression level of the at least one biomarker selected from the group consisting of Bmp2, Daam2, Plaat1 and Prss27, i.e. the indices characteristic of the subject and the reference indices incorporate the expression level of the additional biomarker as well as the at least one biomarker selected from the group consisting of Bmp2, Daam2, Plaat1 and Prss27.

Preferably the expression level(s) and the amount are determinded *in vitro*.

In another aspect a method for diagnosing tissue hypothyroidism in a subject is provided, comprising

i) measuring the level of expression of one or more biomarkers selected from the group consisting of Bmp2, Daam2, Plaat1, Prss27,

AGAP5, ANKRD53, ARMC4, CES3, CPNE9, ERVH48-1, FAM157A, FAM41C, GP2, GTF2IP20, IL3RA, IZUMO4, LAT, LINC00471, LINC01422, MARK2P13, MGAM2, MIR670HG, MSANTD1, NXPE2, PYROXD2, RN7SL600P, SPATA25, SPDYE1, TBXA2R, WFDC12, WWC2-AS2, YJEFN3, ZGLP1

in a hair follicle sample of the subject and

ii) ii) comparing the level of expression measured in i) to a corresponding reference value.

In another aspect a method of treating a subject suffering from tissue hypothyroidism is provided, the method comprising:

i) measuring the level of expression of one or more biomarkers selected from the group consisting of Bmp2, Daam2, Plaat1, Prss27,

AGAP5, ANKRD53, ARMC4, CES3, CPNE9, ERVH48-1, FAM157A, FAM41C, GP2, GTF2IP20, IL3RA, IZUMO4, LAT, LINC00471, LINC01422, MARK2P13, MGAM2, MIR670HG, MSANTD1, NXPE2, PYROXD2, RN7SL600P, SPATA25, SPDYE1, TBXA2R, WFDC12, WWC2-AS2, YJEFN3, ZGLP1

in a hair follicle sample of the subject,

ii) comparing the level of expression measured in i) to a corresponding reference value,

iii) determining whether the subject has tissue hypothyroidism based on the comparison of ii) and

treating the subject by dietary and/or pharmaceutical intervention to restore tissue euthyroidism.

Preferably the one or more biomarkers are selected from the group consisting of Plaat1, Prss27, AGAP5, ANKRD53, ARMC4, CES3, CPNE9, ERVH48-1, FAM157A, FAM41C, GP2, GTF2IP20, IL3RA, IZUMO4, LAT, LINC00471, LINC01422, MARK2P13, MGAM2, MIR670HG, MSANTD1, NXPE2, PYROXD2, RN7SL600P, SPATA25, SPDYE1, TBXA2R, WFDC12, WWC2-AS2, YJEFN3, ZGLP1.

Preferably comparing the level of expression of the at least one biomarker to a corresponding reference value comprises

a) calculating one or more indices characteristic of the subject based on the measured expression level and optionally on the measured amount of the additional biomarker and b) comparing the one or more indices calculated in a) to one or more indices calculated from the level of expression of the at least one biomarker measured in a reference sample.

Preferably the subject is diagnosed as euthyroid based on a serum thyroid function test. Preferably the subject exhibits symptoms of hypothyroidism.

EXAMPLES

Human study

Study design and participants

In this cross-sectional, multicenter study, subjects were enrolled as not amiodarone treated calibrators (CALIB) or amiodarone treated individuals (AMIO). In the CALIB group, HF samples were taken from patients with hypothyroidism or hyperthyroidism at the participating endocrine centers, 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, non-pregnant, had no known inherited disease, including resistance to thyroid hormone. Enrolled subjects were not stratified for ethnicity, lifestyle, comorbidities and background of hypo and hyperthyroidism. In the AMIO group, amiodarone was started at least 4 weeks before HF sampling.

Table 1. Characteristics of human subjects

CALIB	Hypothyroid	Euthyroid	Hyperthyroid	All subjects
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 [mIU/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	Hypothyroid	Euthyroid	Hyperthyroid	All patients
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 [mIU/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

Serum and concomitant HF samples were taken the same day, preferably at the same time. Serum TSH, fT4 and fT3 were measured by 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 subject's vertex skin. The hair follicles were cut from each hair. HF samples were placed immediately after collection in Eppendorf tubes containing 100-300 µl Extraction Buffer of Arcturus PicoPure kit and stored at -20 °C until used. Total RNA was isolated with 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% Et-OH 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 were selected for Next Generation Sequencing in order to identify TH sensitive genes as biomarker candidates.

At first, the RNA concentration and quality (A260/280 ratio) were measured with DeNovix spectrophotometer (DeNovix, Inc., Wilmington, DE, USA) according to the manufacturers' instructions.

Then the RNA samples were subjected to quality check on Agilent BioAnalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA) using Eukaryotic Total RNA Nano Kit according to manufacturer's protocol. Samples with RNA integrity number (RIN) value >7 were further processed for library preparation.

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

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

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

Taqman PCR on tissue biomarker candidates:

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 cDNA was used in Taqman reaction of the following transcripts: Bmp2, Col7a1, Fzd10, Insig1, Itgb4, Kplce, Krt16, Krtap9-7, Prss27, 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, 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.

Statistical analysis of human data

Data was organized with Microsoft Excel. Statistical analysis of human data was done with Tibco STATISTICA v14 and SPSS v23. Both softwares yielded same result. GraphPad Prism 9 and Adobe Illustrator were used to create figures. In order to make figures more comprehensible, gene expression data was normalized to the mean of euthyroid group (ddCt) and multiplied by minus one (–ddCt). One unit increase in –ddCt means twofold increase in gene expression.

Analysis of covariance was used to compare gene expression level of hypothyroid, euthyroid and hyperthyroid 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 analysed 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 one variable. The Kaiser method was applied, and one component was sufficient to explain 81 % of total variance.

Multinomial logistic regression model was built with serum-based status of hypothyroid, euthyroid and hyperthyroid as outcome, principal component of markers, age, and sex assigned at birth (hereinafter: sex) were continuous predictors. Euthyroid group was used as reference. Model was validated with bootstrapping of 1000 samples. Model performance metrics were determined based on confusion matrices and ROC curves (Zaman NID, Hau YW, Leong MC, Al-ashwal RHA. A review on the significance of body temperature interpretation for early infectious disease diagnosis. Artificial Intelligence Review. 2023/12/01 2023;56(12):15449-15494). Predictive model was deployed on AMIO sample, and the confusion matrices of CALIB and AMIO predictions were analyzed with 2x2 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 HF-based prediction coincided with serum-based category of subject. Fisher's corrected $\chi 2$ test with 95 % confidence was used to assess the statistical significance of the variables interdependence. Effect sizes are given as odds ratios, and all confidence intervals are of 95 % confidence levels.

Results

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 1.

Development of a hair follicle-based approach

For assessing human tissue TH signaling, Next Generation Sequencing of HF samples of hypothyroid, euthyroid and hyperthyroid subjects (n= 5/group) revealed 1633 differentially expressed transcripts (Figure 1). However, most of these (e.g. Ccnd1, which had already been indicated in the literature, see Hönes et al. Canonical Thyroid Hormone Receptor β Action Stimulates Hepatocyte Proliferation in Male Mice Endocrinology, Volume 163, Issue 3, March 2022, bqac003) were not suitable for diagnosis using hair follicles.

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 confidence intervals of hypothyroid, euthyroid and hyperthyroid groups. Four genes, bone morphogenic protein 2 (Bmp2), dishevelled 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 2.7-6.5 fold difference in gene expression between hypo- and hyperthyroid cases in this subsample, and were selected as tissue TH markers for further investigation (Figure 2). In another experiment *Wfdc12* and *Yjefn3* also proved to be controlled by thyroid hormones (Figure 7 and 8).

Expression of selected marker genes was measured 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 (Table 2 and 3).

Table 2. Parameter estimates and statistics of the 4-gene (*Bmp2+Daam2+ Plaat1+ Prss27)* prediction model for classifying subjects into hypothyroid, euthyroid, or hyperthyroid. The euthyroid group served as reference group. Abbreviations: CI: confidence interval.

Bmp2+Daam2+ Plaat1+ Prss27		Estimate	Standard Error	Wald statistic	Lower CI 95%	Upper CI 95%	p
Predicting hyperthyroid	Intercept	-0.256	0.783	0.107	-1.790	1.278	0.74
	Marker genes	-0.727	0.180	16.407	-1.079	-0.375	0.0001
	Sex	0.256	0.497	0.266	-0.717	1.229	0.60
	Age	-0.012	0.016	0.568	-0.043	0.019	0.45
Predicting hypothyroid	Intercept	-1.283	0.758	2.867	-2.768	0.202	0.09
	Marker genes	0.491	0.194	6.392	0.110	0.872	0.011
	Sex	0.869	0.464	3.509	-0.040	1.778	0.061
	Age	0.001	0.014	0.007	-0.027	0.029	0.93

Table 3. Confusion matrix of calibrator sample after deploying the 4-gene (*Bmp2+Daam2+ Plaat1+ Prss27)* prediction model for classifying subjects into hypo-, eu-, or hyperthyroid based on novel tissue markers. Observed means serum-based, predicted means tissue marker-based categorization.

Bmp2+Daam2+ Plaat1+ Prss27	Predicted hypothyroid	Predicted euthyroid	Predicted hyperthyroid
Observed hypothyroid	20	17	5

Observed euthyroid	12	39	9
Observed hyperthyroid	1	19	24

The model was validated by bootstrapping and showed good performance characteristics (Table 4 and 5; and Figure 3). This shows that in the CALIB sample serum-based TH status is reflected at the tissue level, and the four 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.

Table 4. Bootstrap validation of the 4-gene prediction model for classifying subjects into hypothyroid, euthyroid, or hyperthyroid based on novel tissue markers. The table shows results of 1000 samples. The euthyroid group served as reference group.

Bmp2+Daam2+ Plaat1+ Prss27		Estimate	Bias	Standard error	95% Confidence Interval		p
					Lower	Upper	
Predicting hyperthyroid	Intercept	-0.256	-0.019	0.967	-2.22	1.72	0.75
	Marker genes	-0.727	-0.053	0.179	-1.18	-0.48	0.001
	Sex	0.256	-0.018	0.528	-0.78	1.31	0.61
	Age	-0.012	0.000	0.019	-0.05	0.02	0.47
Predicting hypothyroid	Intercept	-1.283	-0.068	0.776	-3.02	0.06	0.065
	Marker genes	0.491	0.027	0.230	0.12	0.98	0.015
	Sex	0.869	0.053	0.524	-0.11	2.01	0.074
	Age	0.001	0.000	0.015	-0.03	0.03	0.94

Table 5. Total accuracy and area under the receiver operating characteristic (ROC) curves of the 4-gene prediction model for classifying patients into hypothyroid (hypo), euthyroid (eu), or hyperthyroid (hypo) based on novel tissue markers.

Bmp2+Daam2+ Plaat1+ Prss27	Predict Hypo	Predict Eu	Predict Hyper
Area under curve	0.76	0.66	0.84
Total accuracy of the model	0.57		

The performance of the individual genes (*Daam2*, *Bmp2*, *Plaat1*, *Prsss27*) and the combinations thereof was also investigated.

Table 6. Parameter estimates and statistics of the 3 gene (*Prss27+Daam2+Bmp2*; *Prss27+Daam2+Plaat1; Daam2+Bmp2+Plaat1)* prediction models (CI: confidence interval)

Prss27+Daam2+Bmp2		Estimate	Standard Error	Wald statistic	Lower CI 95%	Upper CI 95%	p
Predicting hyperthyroid	Intercept	-0.217	0.839	0.067	-1.861	1.428	0.796
	Marker genes	-0.752	0.208	13.113	-1.159	-0.345	0.000
	Sex	0.304	0.531	0.328	-0.737	1.345	0.567
	Age	-0.013	0.017	0.632	-0.046	0.019	0.427
Predicting hypothyroid	Intercept	-1.323	0.820	2.604	-2.930	0.284	0.107
	Marker genes	0.590	0.233	6.418	0.133	1.046	0.011
	Sex	0.958	0.512	3.494	-0.046	1.962	0.062
	Age	0.001	0.015	0.002	-0.029	0.031	0.967
P*rss27+Daam2+Plaat1*		Estimate	Standard Error	Wald statistic	Lower CI 95%	Upper CI 95%	p
Predicting hyperthyroid	Intercept	-0.371	0.770	0.232	-1.881	1.139	0.630
	Marker genes	-0.960	0.216	19.664	-1.384	-0.535	0.000
	Sex	0.192	0.488	0.155	-0.765	1.149	0.694
	Age	-0.009	0.016	0.351	-0.040	0.021	0.554
Predicting hypothyroid	Intercept	-1.223	0.725	2.846	-2.643	0.198	0.092
	Marker genes	0.481	0.206	5.448	0.077	0.886	0.020
	Sex	0.762	0.437	3.041	-0.094	1.618	0.081
	Age	0.002	0.014	0.023	-0.025	0.029	0.881
Daam2+Bmp2+Plaat1		Estimate	Standard Error	Wald statistic	Lower CI 95%	Upper CI 95%	p
Predicting hyperthyroid	Intercept	-0.167	0.755	0.049	-1.647	1.313	0.825
	Marker genes	-0.723	0.188	14.746	-1.091	-0.354	0.000
	Sex	0.352	0.482	0.535	-0.592	1.297	0.464
	Age	-0.014	0.015	0.813	-0.043	0.016	0.367
Predicting hypothyroid	Intercept	-1.244	0.744	2.797	-2.702	0.214	0.094
	Marker genes	0.490	0.217	5.125	0.066	0.915	0.024
	Sex	0.828	0.453	3.336	-0.060	1.716	0.068
	Age	0.002	0.014	0.024	-0.025	0.030	0.877

Table 7. Performance metrics of the 3 gene prediction models ((*Prss27+Daam2+Bmp2*; *Prss27+Daam2+Plaat1; Daam2+Bmp2+Plaat1;* values are probabilities of specific, individual classification and are ranging from 0 to 1, higher is better). Metrics were calculated based on Zaman NID, Hau YW, Leong MC, Al-ashwal RHA. A review on the significance of body 512 temperature interpretation for early infectious disease diagnosis. Artificial Intelligence Review. 513 2023/12/01 2023;56(12):15449-15494. (Hypo=hypothyroid, Eu= euthyroid, Hyper= hyperthyroid)

Prss27+Daam2+Bmp2	Predict Hypo	Predict Eu	Predict Hyper
Precision	0.86	0.70	0.86
Sensitivity	0.79	0.80	0.80
Specificity	0.83	0.64	0.82
Negative predictive value	0.74	0.75	0.75
Area under curve	0.76	0.67	0.82
Total accuracy of the model	0.57		
Prss27+Daam2+Plaat1	Predict Hypo	Predict Eu	Predict Hyper
Precision	0.87	0.69	0.87
Sensitivity	0.78	0.81	0.81
Specificity	0.83	0.63	0.84
Negative predictive value	0.72	0.76	0.76
Area under curve	0.74	0.68	0.82
Total accuracy of the model	0.57		
Daam2+Bmp2+Plaat1	Predict Hypo	Predict Eu	Predict Hyper
Precision	0.86	0.68	0.86
Sensitivity	0.77	0.81	0.80
Specificity	0.83	0.63	0.83
Negative predictive value	0.73	0.77	0.75
Area under curve	0.74	0.66	0.81
Total accuracy of the model	0.56		
Prss27+Bmp2+Plaat1	Predict Hypo	Predict Eu	Predict Hyper
Precision	0.89	0.71	0.88
Sensitivity	0.80	0.84	0.81
Specificity	0.84	0.62	0.83
Negative predictive value	0.73	0.78	0.75
Area under curve	0.76	0.66	0.83

Total accuracy of the model	0.60

Table 8. Parameter estimates and statistics of the prediction model based on *Prss27, Bmp2, Plaat1 or Daam2* (CI: confidence interval)

Prrs27		Estimate	Standard Error	Wald statistic	Lower CI 95%	Upper CI 95%	p
Predicting hyperthyroid	Intercept	-1.172	0.856	1.872	-2.850	0.507	0.171
	Marker genes	-0.842	0.191	19.443	-1.217	-0.468	0.000
	Sex	0.187	0.503	0.138	-0.800	1.173	0.711
	Age	-0.010	0.016	0.401	-0.041	0.021	0.526
Predicting hypothyroid	Intercept	-0.873	0.739	1.398	-2.321	0.574	0.237
	Marker genes	0.416	0.152	7.501	0.118	0.714	0.006
	Sex	0.838	0.461	3.306	-0.065	1.740	0.069
	Age	0.001	0.014	0.006	-0.026	0.028	0.939
Bmp2		Estimate	Standard Error	Wald statistic	Lower CI 95%	Upper CI 95%	p
Predicting hyperthyroid	Intercept	1.374	0.895	2.355	-0.381	3.129	0.125
	Marker genes	-0.432	0.169	6.577	-0.763	-0.102	0.010
	Sex	0.555	0.476	1.363	-0.377	1.487	0.243
	Age	-0.019	0.015	1.752	-0.048	0.009	0.186
Predicting hypothyroid	Intercept	-2.872	1.089	6.959	-5.005	-0.738	0.008
	Marker genes	0.537	0.211	6.502	0.124	0.950	0.011
	Sex	0.956	0.475	4.049	0.025	1.886	0.044
	Age	0.002	0.014	0.014	-0.026	0.030	0.905
Plaat1		Estimate	Standard Error	Wald statistic	Lower CI 95%	Upper CI 95%	p
Predicting hyperthyroid	Intercept	1.632	0.815	4.008	0.034	3.230	0.045
	Marker genes	-0.763	0.177	18.574	-1.110	-0.416	0.000
	Sex	0.305	0.475	0.413	-0.625	1.235	0.520
	Age	-0.011	0.015	0.544	-0.040	0.018	0.461
Predicting hypothyroid	Intercept	-1.606	0.839	3.662	-3.251	0.039	0.056
	Marker genes	0.195	0.160	1.489	-0.118	0.508	0.222
	Sex	0.598	0.418	2.044	-0.222	1.418	0.153
	Age	0.006	0.013	0.192	-0.020	0.032	0.661

Daam2		Estimate	Standard Error	Wald statistic	Lower CI 95%	Upper CI 95%	p
Predicting hyperthyroid	Intercept	0.572	0.783	0.533	-0.964	2.107	0.466
	Marker genes	-0.525	0.137	14.746	-0.793	-0.257	0.000
	Sex	0.436	0.489	0.793	-0.523	1.394	0.373
	Age	-0.015	0.016	0.914	-0.045	0.016	0.339
Predicting hypothyroid	Intercept	-1.516	0.785	3.728	-3.056	0.023	0.054
	Marker genes	0.268	0.149	3.254	-0.023	0.559	0.071
	Sex	0.742	0.452	2.691	-0.145	1.629	0.101
	Age	0.003	0.014	0.047	-0.024	0.030	0.829

Table 9. Performance metrics of the 1 gene prediction models (values are probabilities of specific, individual classification and are ranging from 0 to 1, higher is better). (Hypo=hypothyroid, Eu= euthyroid, Hyper= hyperthyroid)

prss27	Predict Hypo	Predict Eu	Predict Hyper
Precision	0.88	0.73	0.89
Sensitivity	0.80	0.84	0.85
Specificity	0.82	0.63	0.83
Negative predictive value	0.71	0.77	0.78
Area under curve	0.76	0.70	0.85
Total accuracy of the model	0.62		
Bmp2	Predict Hypo	Predict Eu	Predict Hyper
Precision	0.86	0.74	0.83
Sensitivity	0.82	0.82	0.79
Specificity	0.81	0.67	0.78
Negative predictive value	0.76	0.76	0.73
Area under curve	0.73	0.65	0.74
Total accuracy of the model	0.58		
Plaat1	Predict Hypo	Predict Eu	Predict Hyper
Precision	0.88	0.65	0.82
Sensitivity	0.71	0.80	0.81
Specificity	0.86	0.63	0.80
Negative predictive value	0.69	0.77	0.79
Area under curve	0.69	0.68	0.82
Total accuracy of the model	0.53		
Daam2	Predict Hypo	Predict Eu	Predict Hyper
Precision	0.83	0.62	0.86
Sensitivity	0.72	0.78	0.76
Specificity	0.83	0.61	0.86
Negative predictive value	0.71	0.78	0.76

Area under curve	0.72	0.69	0.81
Total accuracy of the model	0.50		

Table 10. Model parameters of the 5-gene (*Bmp2+Daam2+Plaat1+Prss27+Wfdc12)* prediction model for classifying subjects into (tissue) hypothyroid, euthyroid, or hyperthyroid. The euthyroid group served as reference group. Abbreviations: CI: confidence interval.

Bmp2+Daam2+ Plaat1+Prss27+ Wfdc12		Estimate	Standard Error	Wald statistic	Lower CI 95%	Upper CI 95%	p
Predicting hypothyroid	Intercept	-0.596966	0.886747	0.45321	-2.33496	1.141026	0.500814
	Marker genes	0.727045	0.240054	9.17287	0.25655	1.197542	0.002456
	Sex	0.247720	0.277704	0.79572	-0.29657	0.792010	0.372378
	Age	-0.009228	0.017919	0.26524	-0.04435	0.025892	0.606545
Predicting hyperthyroid	Intercept	-0.479210	0.935389	0.26246	-2.31254	1.354118	0.608433
	Marker genes	-0.787648	0.226331	12.11095	-1.23125	-0.344048	0.000501
	Sex	-0.098149	0.305896	0.10295	-0.69769	0.501396	0.748317
	Age	-0.005564	0.019451	0.08182	-0.04369	0.032560	0.774846

Table 11. Performance metrics of the 5 gene prediction model for classifying patients into (tissue) hypothyroid, euthyroid, or hyperthyroid based on novel tissue markers. (Hypo=hypothyroid, Eu= euthyroid, Hyper= hyperthyroid)

Bmp2+Daam2+Plaat1+Prss27+Wfdc12	Predict Hypo	Predict Eu	Predict Hyper
Precision	0.88	0.72	0.91
Sensitivity	0.80	0.84	0.84
Specificity	0.82	0.61	0.84
Negative predictive value	0.71	0.77	0.77
Area under curve	0.80	0.70	0.84
Total accuracy of the model	0.62		

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

Next, we deployed our prediction model to predict tissue TH status of AMIO patients (Table 12).

Table 12. Confusion matrix of the 4 gene and the 5 gene prediction model on AMIO sample after deploying the 4 gene or the 5 gene prediction model for classifying patients into hypo-, eu-, or hyperthyroid based on novel tissue markers. Observed means serum-based, predicted means tissue marker-based categorization.

Bmp2+Daam2+ Plaat1+ Prss27	Predicted hypothyroid	Predicted euthyroid	Predicted hyperthyroid
Observed hypothyroid	6	14	1
Observed euthyroid	9	34	2
Observed hyperthyroid	2	14	6
Bmp2+Daam2+ Plaat1+ Prss27+ Wfdc12			
Observed hypothyroid	13	14	2
Observed euthyroid	8	32	4
Observed hyperthyroid	1	11	20

To investigate if amiodarone impacts the concordance of serum- and HF-based classifications, the confusion matrices of the two samples were combined into 2x2 contingency tables where one variable is amiodarone treatment, and the other is if serum-based and HF-based TH statuses coincide, i.e. if serum-based status is reflected at the tissue level (Table 13 and Figure 4).

Table 13. Contingency tables and statistics of human data. Contingency tables of comparing AMIO and CALIB samples. The tables were analyzed with Fisher's corrected $\chi 2$ test with 95 % confidence. Prediction was considered correct if HF-based prediction coincided with serum-based category of subject. Abbreviations: CI: confidence interval; OR: odds ratio.

Hypothyroid	Prediction correct	Prediction incorrect
AMIO	6	15
CALIB	20	22
p	0.18	
OR	0.44	
95% CI	0.14-1.35	
Euthyroid	Prediction correct	Prediction incorrect
AMIO	34	11
CALIB	39	21
p	>0.99	
OR	1.66	
95% CI	0.70-3.94	
Hyperthyroid	Prediction correct	Prediction incorrect
AMIO	6	16
CALIB	24	20

p	0.041	
OR	0.31	
95% CI	0.10-0.95	
All samples	Prediction correct	Prediction incorrect
AMIO	46	42
CALIB	83	63
p	0.50	
OR	0.83	
95% CI	0.49-1.41	

In hypothyroid and euthyroid cases, amiodarone treatment had no significant effect on the co-incidence of serum-based and HF-based prediction of TH status. However, in hyperthyroid AMIO patients, 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 to hyperthyroid CALIB patients. 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. This suggests that amiodarone treatment attenuates tissue TH action under hyperthyroid conditions, and AIH patients are less likely to manifest tissue hyperthyroidism than other hyperthyroid patients.

Animal study

Animals and treatments

We used the Thyroid Hormone Action Indicator (THAI) transgenic mouse model for our animal studies (Mohacsik P, Erdelyi F, Baranyi M, et al. A Transgenic Mouse Model for Detection of Tissue-Specific Thyroid Hormone Action. Endocrinology. Feb 1 2018;159(2):1159-1171. doi:10.1210/en.2017-00582). 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 *Dio*1 promoter, which allows quantitative assessment of TH action in tissues by eliminating the influence of other response elements harboured by endogenous genes.

Studies were performed in a split-plot design with one-way treatment structure for both experimental units; outlined in Figure 5A. Male, 2-3 months old THAI mice were used and 2-4

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 in 1.35 g/kg concentration (custom order from SSniff Specialdiäten GmbH; amiodarone hydrochloride obtained from Merck) for eight weeks, while control animals received normal chow diet. On the last two days of the experiment, animals of each cage were randomly allocated into the vehicle or T4 group and received vehicle or 166.7 ng/bwg T4 i.p (n= 12-13/group) (Mohacsik P, Erdelyi F, Baranyi M, et al. A Transgenic Mouse Model for Detection of Tissue-Specific Thyroid Hormone Action. Endocrinology. Feb 1 2018;159(2):1159-1171. doi:10.1210/en.2017-00582). 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 (Sinko R, Mohacsik P, Kovari 517 D, et al. Different Hypothalamic Mechanisms Control Decreased Circulating Thyroid Hormone Levels in Infection and Fasting-Induced Non-Thyroidal Illness Syndrome in Male Thyroid Hormone Action Indicator Mice. Thyroid. Jan 2023;33(1):109-118. doi:10.1089/thy.2022.0404). 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, whichafter cDNA concentration of products was measured with Qubit ssDNA assay kit (Invitrogen). All Taqman reactions were assayed with 50 ng cDNA on a Viia7 instrument (Applied 252 Biosystems). Hprt1 showed low variability and no significant difference between treatment groups and was used as reference gene. The Taqman gene expressions assays are listed in Table 2 (Thermo Fisher Scientific).

Statistical analysis for animal studies

Data was 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 analysed with two-way crossed analysis of variance (ANOVA) followed by 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 was

normalized to the mean of vehicle on control diet group and multiplied by minus one (-ddCt). One unit increase in –ddCt means a twofold increase in gene expression.

Results

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 eight weeks. Half of the mice in each diet group received vehicle injection, while the other half were made hyperthyroid by T4 injections on the last two days of the experiment.

T4 treatment greatly increased serum fT4 level in animals on either diet, indicating hyperthyroidism (Figure 6A). Increased fT4 level lead to increased Luciferase expression, the marker of TH signaling in THAI mice (Mohacsik P, Erdelyi F, Baranyi M, et al. A Transgenic Mouse Model for Detection of 509 Tissue-Specific Thyroid Hormone Action. Endocrinology. Feb 1 2018;159(2):1159-1171. 510 doi:10.1210/en.2017-00582) in the lungs, a tissue known to be impacted by amiodarone, and in the highly TH-responsive brown adipose tissue (BAT) Silva JE, Larsen PR. Adrenergic activation of triiodothyronine production in brown 533 adipose tissue. Nature. Oct 20-26 1983;305(5936):712-3. Under euthyroid conditions, amiodarone treatment had no effect on local TH action in either tissues. In contrast, amiodarone prevented T4-induced increase of TH signaling (Figure 5B,C). This indicates that amiodarone inhibits the tissue effects of T4 under hyperthyroid conditions by mitigating the development and severity of tissue hyperthyroidism in multiple tissues, suggesting a global, not tissue-specific phenomenon.

CLAIMS

1. A method for determining whether the tissue thyroid status of a human subject is euthyroid, hypothyroid or hyperthyroid, comprising
i) measuring the level of expression of at least one biomarker selected from the group consisting of Bmp2, Daam2, Plaat1, Prss27, Wfdc12 and Yjefn3 in a hair follicle sample of the subject,
ii) comparing the level of expression measured in i) to a corresponding reference value characteristic of an euthyroid, hypothyroid or hyperthyroid sample.

2. The method according to claim 1, wherein the at least one biomarker is selected from Prss27, Wfdc12 and Yjefn3.

3. The method according to claim 2, wherein the at least one biomarker is selected from Prss27 and Wfdc12.

4. The method according to claim 3, wherein the at least one biomarker is Wfdc12.

5. The method according to claim 2, wherein the at least one biomarker is Yjefn3.

6. The method according to any one of claims 1-3, wherein the at least one biomarker is Prss27.

7. The method according to claim 1, wherein the at least one biomarker is Bmp2, Daam2, Plaat1 and Prss27.

8. The method according to claim 1 or 2, wherein the at least one biomarker is Bmp2, Daam2, Plaat1, Wfdc12 and Prss27.

9. The method according to any one of the preceding claims, wherein the subject is/has been diagnosed as hyperthyroid or as hypothyroid using a serum thyroid function test.

10. The method according to claim 9, wherein the subject exhibits mild or no symptoms of hyperthyroidism or hypothyroidism, respectively.

11. The method according to any one of the preceding claims, wherein the subject receives or has received a substance that is capable of inducing or induces hyperthyroidism or hypothyroidism.

12. The method according to claim 11, wherein the subject has been diagnosed as hyperthyroid or as hypothyroid using a serum thyroid function test after receiving the substance.

13. The method according to claim 11 or 12, wherein the substance is amiodarone.

14. The method according to claim 1, wherein
the at least one biomarker is Bmp2, Daam2, Plaat1 and Prss27, and
the subject is diagnosed with amiodarone induced hyperthyroidism using a serum thyroid function test.

15. The method according to any one of the preceding claims, wherein comparing the level of expression of the at least one biomarker comprises
a) calculating one or more indices characteristic of the subject based on the measured expression level and
b) comparing the one or more indices calculated in a) to one or more indices calculated from the level of expression of the at least one biomarker measured in a reference sample.