REVIEW

Biomarkers indicating tissue thyroid hormone status: ready to be implemented yet?

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Abstract

Currently, thyroid hormone status is predominantly determined by the measurement of serum thyroid-stimulating hormone and free thyroxine. Although it is assumed that serum thyroid hormone (TH) concentrations within the reference range represent euthyroidism, it is unknown whether this reflects euthyroidism in all tissues (e.g. brain, muscle, bone and liver). To date, no serum marker has been established for clinical use that represents TH status within tissues accurately. However, several biomarkers have been investigated and innovative techniques have been used to unravel new biomarkers. This review provides an overview of proposed serum biomarkers that reflect tissue TH status in humans. Furthermore, we discuss the feasibility of these serum markers in clinical practice.

Introduction

Thyroid hormone (TH) is essential for development, growth and metabolism; a large variety of cells and organs are affected by TH and dependent on TH for proper function. The main TH target organs are the liver, bone, kidney, intestine and heart, and key functions of these organs are affected by a shortage or excess of TH. In addition, several systems such as the complement and coagulation system are also dependent on TH. Thyroid dysfunction (both overt and subclinical thyroid disease) is one of the most common endocrine disorders with a prevalence of approximately 3.8% in Europe (Garmendia Madariaga et al. 2014). Currently, measuring serum thyroid-stimulating hormone (TSH) and/or free thyroxine (fT4) is leading in establishing TH status. However, it can be questioned whether both TSH and/or fT4 entirely capture TH status throughout the whole body. Furthermore, many patients with hypothyroidism are bound to TH medication (levothyroxine (L-T4)) whose dosage is, depending on the cause, based on TSH or fT4 concentrations measured in blood. Still, a substantial part of those patients experiences discomfort while serum TSH or fT4 concentrations are within the reference interval. Serum TSH, as part of the hypothalamus–pituitary–thyroid axis, regulates the production of thyroxine (T4) and triiodothyronine (T3) by the thyroid gland. In this review, TH refers to both fT4, free triiodothyronine (fT3), total T4 and total T3 and specified if needed. The prohormone T4 is the most abundant TH in the circulation and fT4 is taken up by the cell via specific TH transporters. Within the cell, T4 is metabolized by TH converting enzymes (deiodinases) toward T3. T3 is the active hormone which predominantly exerts its action via binding to the...
TH receptor (TR). In clinical practice, it is assumed that serum TH concentrations reflect TH status in the tissues. However, previous research in both humans and rats indicates that serum TH concentrations may not always correspond with tissue TH status (Escobar-Morreale et al. 1995, Dumitrescu & Refetoff 2013). This might be due to changes in TH transport across the cell, TH metabolizing enzymes or T3-TR binding.

To investigate true TH action in an organ, a tissue biopsy is needed to measure tissue TH status or establish TH regulated pathways. Since this is not feasible in clinical practice and if feasible, TH tissue measurements are not validated in all tissues, there is a need for accurate serum markers representing (specific) tissue TH status. These serum markers are useful for several purposes. First, the determination of serum markers of tissue TH status might improve treatment. Ideally, these markers should give a better representation of euthyroidism in tissues than the currently used serum TSH and/or fT4 concentrations. Subsequently, treatment can be targeted to improve, for example, cardiovascular outcomes, osteoporosis, or overall well-being. It might help to understand why patients treated with L-T4 still experience discomfort even though TSH and/or fT4 concentrations are within the reference interval. Furthermore, TH therapy such as TR agonists, for treating other conditions than thyroid disorders (e.g. hepatosteatosis), could be monitored more easily using serum markers of tissue TH status. Finally, these markers could be used to improve diagnostics. Some thyroid disorders cannot be differentiated based on TSH and/or fT4 concentrations (such as resistance to TR (RTH) and TSH-secreting adenoma), in that case, biomarkers reflecting tissue TH status might be discriminating.

Over the past decade, several metabolic biomarkers have been associated with TH action (e.g. cholesterol, osteocalcin and sex hormone-binding globulin (SHBG)). Currently, innovative techniques using omics are used to examine not only one marker but determine multiple markers in order to unravel a panel of markers representing (tissue) TH status. Metabolomic profiling is a powerful tool for measuring the activity of metabolic tissues (Newgard 2017). Thousands of small molecule metabolites can be measured to represent the current metabolic state of specific cells, tissues or whole organisms (Smith et al. 2006). Targeted and nontargeted metabolomics can be distinguished. Targeted metabolomics is used when the specific concentration of a known metabolite is of interest. Nontargeted metabolomics are best suited to identify new metabolites affecting specific pathways. Metabolomics has been used so far to establish markers in different types of diabetes, cardiovascular diseases, fatty liver, non-alcoholic steatohepatitis (NASH), and TH status (Newgard 2017, Friedrich et al. 2020). Along with metabolomics, proteomic profiling has been used as well, where instead of metabolites, proteins are determined. Interestingly, associations with more classical markers of TH action previously described are also found when using these techniques.

Pathways in human TH metabolism

Tissue TH availability is determined by an interplay between serum TH levels, TH transporters, and cellular TH metabolizing enzymes, which is shown in a simplified manner in Fig. 1 and explained below. Once produced by the thyroid gland, TH is released into the bloodstream and transported to the tissues mostly bound to thyroid-binding globulin. Then, TH actively enters the cell via TH transporters. Monocarboxylate transporters (MCT8, MCT10), organic anion-transporting polypeptide (OATP1c1) and solute carrier family (SLC17A4) transporters are thought to facilitate this transport most efficiently (Friesema et al. 2006, 2008, van der Deure et al. 2008, Teumer et al. 2018). MCT8 has a better affinity for the transport of T3, where MCT10 and OATP1c1 transport predominantly T4 into the cells. Transporters are also involved in the transport of T3 out of the cell. Not all transporters are expressed in all tissues. MCT8 is expressed in neurons, the pituitary, liver, kidney, and adipose tissue, while MCT10 is expressed in the kidney, liver, muscle, macrophages, and hypothalamus. OATP1c1 is expressed in brain capillaries and therefore involved in the transportation of T4 over the blood-brain barrier and SLC17A4 is predominantly expressed in small intestinal and colonic epithelial cells, pancreas, liver and kidney.

In the cell, T4 and T3 are subject to pathways that activate or inactivate them. Three major peripheral pathways are described and are important in determining cellular TH status: deiodination, sulfation and glucuronidation. Other pathways (deamination and decarboxylation of the alanine side chain of TH, ether-link cleavage) also play a role in the TH metabolism, but their contribution is minor.

It is known that deiodination contributes greatly to the conversion of T4 into the biologically active T3 through outer-ring deiodination and to the degradation of T4 and T3 to respectively reversed T3 (rT3) and the inactive TH metabolite 3,3’-diodothyronine (T2) through inner ring deiodination. Three types of deiodinases (DIO1, DIO2 and DIO3) are known and have distinct functions in the
(in)activation of TH (van der Spek et al. 2017). DIO1 is able to perform both inner- and outer-ring deiodination. Its main role is the degradation of inactivated TH; however, DIO1 also plays a role in the conversion of T4 into the biologically active T3, especially in the liver. DIO2 has predominantly an activation role and is only capable of outer-ring deiodination and prefers deiodinating T4 into T3. The function of DIO3 is to inactivate TH, since it is only capable of inner-ring deiodination, DIO3 catalyzes the conversion of T3 into T2 and T4 into rT3. Interestingly, not all types are present in the same tissue. DIO1 is located in the liver, kidney, thyroid, and pituitary, whereas DIO2 is among others expressed in many brain areas, pituitary, brown adipose tissue, placenta, skeletal muscle, and macrophages. DIO3 is predominantly present in the placenta and neurons in the brain and plays a role in fetal development (Köhrl 2000). Genetic variation in deiodinases is common (Taylor et al. 2015) and previous research in deiodinase knock-out mice proved aberrant serum TH concentrations compared to wild-type (WT) mice. In general, deiodinases contribute largely to TH tissue status and defects are of interest for the regulation of TH concentrations in specific tissues.

The pathway of sulfation interacts with the deiodination pathway just described. Sulfotransferases sulfate both T4 and T3. Sulfation of T4 (T4S) blocks the outer-ring deiodination, which disables the conversion of T4 into active T3 facilitated by DIO1. Subsequently, the only possible conversion, of T4S to rT3S, leads to an irreversible inactivation of TH. T4S is in fact a preferred substrate for DIO1 which subsequently leads to accelerated degradation of T4S into rT3S (Visser 1994). On the other hand, sulfated T3 (T3S) is reversible, which enables T3S to serve as T3 storage.

Another metabolic pathway, predominantly for T4, is glucuronidation. Glucuronidated T4 (T4G) can serve as a reservoir as well, predominantly in the gut, where β-glucuronidase can deconjugate T4G back to T4. Some medications (such as antiepileptic medication) are glucuronidation-inducing, which can lead to decreased serum T4 levels. Although this does not cause clinical hypothyroidism in initially euthyroid people, it can increase the need for a higher dosage of T4 supplementation in hypothyroid patients (Bianco & Kim 2013).

Once T4 is converted into its active form T3, TRs are primarily necessary to establish the actual effect of T3 either with or without (in)direct DNA binding. Furthermore, T3 can exert its effect independent of TRs via integrins (Flamant et al. 2017, Davis et al. 2021). Three TRs that bind T3 are known; TRα1, TRβ1 and TRβ2 which are encoded by the THRA and THRβ genes. TRα1 is predominantly expressed in the brain, heart, spleen, intestine and bone, ...
whereas TRβ1 is mostly expressed in the liver and TRβ2 in the pituitary, paraventricular nucleus of the hypothalamus, retina, and the inner ear. The effects of T3 can roughly be achieved via two pathways: the canonical or noncanonical pathway. The canonical pathway is the ‘classic’ pathway and reaches its effect through gene expression (Yen 2001). TRs bind to TH-responsive elements (TREs) and binding of T3 to TRs promotes coactivators which enhances gene transcription. In the absence of T3, corepressors are active, which reduces gene transcription. An additional pathway that does not require binding to DNA and TREs and where TRs rapidly activate intracellular second-messenger signaling pathways (e.g. PI3K pathway) without gene expression is called noncanonical. The study of Hönes et al. (2017) showed that several TH-dependent physiological effects stay intact in mice with total loss of the canonical pathway. This indicates that TR binding to DNA and TREs can be bypassed via the noncanonical pathway. It also indicates that T3 activates genes in the absence of the classic canonical pathway.

TH transporters, deiodinases and TRs are obviously of great importance to the availability of cellular TH and therefore the effect of TH. Changes in these components can lead to an imbalance between the available serum TH and cellular TH. A few conditions that highlight the necessity to be able to discriminate between serum TH concentrations and specific tissue TH concentrations will be discussed briefly. Mutations in the THRA and THRβ genes cause distinct genetic diseases called RTHα and RTHβ. RTHα causes hypothyroidism in TRα-dominant tissues (intestine, bone, heart and brain), whereas serum TH concentrations are relatively normal. This complicates the diagnosis, while it is important to quickly recognize this disease because developmental consequences can be greatly reduced by early treatment with L-T4 (Dumitrescu & Refetoff 2013, van Gucht et al. 2016). An imbalance in TH concentration is also seen in non-thyroidal illness syndrome (NTIS). NTIS is characterized by low serum T4 and T3 concentrations and normal TSH concentrations. During acute inflammation, T3 availability in skeletal muscles is increased, whereas liver T3 concentrations seem to be reduced (Boelen et al. 2011) indicating a disbalance in TH concentrations between the circulation and a variety of organs. However, there are still many uncertainties and it might be of clinical importance to be able to determine tissue TH status during illness to consider appropriate treatment. Another example is the presence of a mutation in the gene coding for the TH transporter MCT8 leading to an x-linked disease which is associated with Allan–Herndon–Dudley syndrome. This disease presents with normal/slightly increased TSH, decreased serum T4, increased serum T3 and decreased serum rT3. Tissue TH concentrations are, however, varying between different tissues. The MCT8 mutation causes the inability to transport T3 and T4 into cells that express MCT8. Cells that do not express MCT8 but a compensating transporter are subject to high T3 and, therefore, hyperthyroid, likewise cells that express MCT8 without compensating transporter are hypothyroid (Friesema et al. 2010). Determining TH status per tissue would be helpful in this situation.

**Tissue-specific metabolic markers**

Over the years, several (tissue-specific) markers have been distinguished which might correlate with human thyroid status. Nowadays, innovative techniques that have been developed enhance the ability to discover new biomarkers (Pietzner et al. 2018). In this review, we give an up-to-date overview of different markers per tissue in humans including the strengths and limitations (Table 1).

**Liver**

The liver is an important TH target tissue with several metabolic functions being TH-dependent, such as lipid and carbohydrate metabolism and therefore regulating energy balance (Sinha et al. 2019). It expresses mainly TRβ1, although TRβ2 is also expressed to a lesser degree and is heavily involved in thyroid metabolism via high expression of DIO1. Several liver-related conditions, like hypercholesterolemia and nonalcoholic fatty liver disease, have been associated with TH status (Sinha et al. 2019).

**Lipids and cholesterol**

THs are highly involved in the regulation of lipid and cholesterol metabolism via various mechanisms; T3 increases the transcription rate of genes encoding for enzymes involved in lipogenesis and beta-oxidation, and induces HMG-CoA reductase in the liver which in turn stimulates cholesterol synthesis (Cachefo et al. 2001, Sinha et al. 1999). Overall, hypothyroidism resulted in an increase in concentrations of LDL, HDL, total cholesterol, Apo B, and triglycerides, whereas hyperthyroidism was characterized by a decrease in predominantly LDL and total cholesterol concentrations (Nishitani et al. 1990, Kung et al. 1995, Sundaram et al. 1997, Erem et al. 1999, 2015, Altınova 2006). The other markers showed inconsistent results. Restoring euthyroidism resulted in changes of these markers toward concentrations comparable to healthy euthyroid controls.
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<td>Liver</td>
<td>Lipids</td>
<td>Single measurement and MS/NMR-derived metabolomics</td>
<td>Increased in hyperthyroidism; effect independent of influencing factors; careful interpretation.</td>
<td>Pietschner et al. 2017, Boumaza et al. 2019</td>
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<td></td>
<td>GPDH</td>
<td>Single measurement</td>
<td>Increased in hyperthyroidism; cannot be measured in blood; metabolic substrate of GPDH; research is still scarce.</td>
<td>Lee et al. 1959, Lee &amp; Lardy 1965, Okamura et al. 1981</td>
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<td></td>
<td>Glycerol</td>
<td>NMR-metabolomics</td>
<td>Increased in hypothyroidism in one study; can be measured in blood; strongly influenced by inflammation and iron status.</td>
<td>Piras et al. 2021</td>
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<td></td>
<td>CD5L</td>
<td>Multi-omics analysis</td>
<td>Positively correlated with fT3 in hyperthyroid state; influences by other factors have to be investigated.</td>
<td>Nock et al. 2020</td>
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<td></td>
<td>SHBG</td>
<td>Single measurement</td>
<td>Positively associated with fT3 and fT4; helpful in prediction of outcome resmetirom on NASH; interferences should be taken into account.</td>
<td>Dumoulin et al. 1995, Vierhapper et al. 1998, Brenta et al. 1999, Hampl et al. 2003, Heijboer et al. 2016, Ito et al. 2017</td>
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<td></td>
<td>Ferritin</td>
<td>Single measurement</td>
<td>Increased in hyperthyroidism and decreased in hypothyroidism; large between-individual variation; strongly influenced by inflammation and iron status.</td>
<td>Kubota et al. 1993, , Sachdeva et al. 2015</td>
</tr>
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<td>Cardiovascular</td>
<td>SCUBE1</td>
<td>Single measurement</td>
<td>Increased in both hypo- and hyperthyroidism.</td>
<td>Bilir et al. 2016, Erem et al. 2016</td>
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<td></td>
<td>t-PA</td>
<td>Single measurement</td>
<td>Decreased in hyperthyroidism; inversely correlated with T3 and T4 in hyperthyroid state; markedly influenced by other factors.</td>
<td>Li et al. 1998</td>
</tr>
<tr>
<td></td>
<td>PAI-1</td>
<td>Single measurement</td>
<td>Positively correlated with TH in hyperthyroid state; markedly influenced by other factors.</td>
<td>Li et al. 1998</td>
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<td></td>
<td>Coagulation proteins</td>
<td>Proteomic analysis</td>
<td>Relation between proteins involved in the coagulation cascade and TH; markedly influenced by other factors.</td>
<td>Engelmann et al. 2015, Alfadda et al. 2018</td>
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<td></td>
<td>ACE</td>
<td>Single measurement</td>
<td>Increased in hyperthyroidism and decreased in hypothyroidism; markedly influenced by other factors.</td>
<td>Smallridge et al. 1983, Graninger et al. 1986, Lee et al. 1986, Reiners et al. 1988</td>
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<td>Bone alkaline phosphatase</td>
<td>Single measurement</td>
<td>Positively correlated with fT3 and fT4; no deviant outcomes in hypothyroidism</td>
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<td>P1NP</td>
<td>Single measurement</td>
<td>Increased in hyperthyroid state and decreased in hypothyroid state; more research needed</td>
<td></td>
<td>Kužma et al. 2018, Mat Ali et al. 2020</td>
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<tr>
<td>TRAP-5b</td>
<td>Single measurement</td>
<td>Inconsistent outcomes in hyperthyroid and hypothyroid patients</td>
<td></td>
<td>Jódar et al. 1997, Minisola et al. 2002</td>
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<tr>
<td>Ca</td>
<td>Single measurement</td>
<td>Increased in hyperthyroidism; hypothyroidism did not cause deviant outcomes; strongly influenced by other conditions</td>
<td></td>
<td>Mosekilde &amp; Christensen 1977, Kobe et al. 1999, Sabuncu et al. 2001, Kisakol et al. 2003, Dhanwal 2011</td>
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<tr>
<td>Intestine</td>
<td>Intestinal alkaline phosphatase</td>
<td>Single measurement</td>
<td>Decreased in hypothyroid state; hypothyroid state has not been investigated yet</td>
<td>Watson &amp; Tuckerman 1971, Hodin et al. 1996, Malo et al. 2004</td>
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<td>Non-organ specific markers</td>
<td>Cu</td>
<td>Single measurement</td>
<td>Positively correlated with fT4, T3 and T4; strongly influenced by other factors</td>
<td>Mittag et al. 2012, Jain 2014, Blasig et al. 2016</td>
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(Continued)
(Muls et al. 1982, Klausen et al. 1992, Arem et al. 1995, Hoppickler et al. 1995, Kung et al. 1995, Hayashi et al. 1996, O’Brien et al. 1997, Martínez-Triguero et al. 1998, Ozata et al. 1998, Paoli et al. 1998, Becerra et al. 1999, Tzotzas et al. 2000, Ito et al. 2007, Erem et al. 2015). Therefore, a serum lipid and cholesterol profile has been shown to be a marker of TH action. Research in thyroidectomized patients using L-T4 showed that biochemical euthyroidism (defined by TSH within the reference range) while treated may be associated with increased cholesterol concentrations (Lee et al. 2019). This strengthens the assumption that cholesterol could be of interest as a biomarker to determine liver TH status. Innovative techniques confirmed the association between lipids, cholesterol and TH status. Analysis via untargeted NMR-derived metabolomics demonstrated that hypothyroid mice had decreased concentrations of VLDL and LDL and increased concentrations of unsaturated lipids and cholesterol compared to euthyroid

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<tr>
<td>Se/Cu ratio</td>
<td>Single measurement</td>
<td>Increased in RTHβ patients compared to patients with TSH-secreting pituitary adenoma; could serve as a biomarker to differentiate between these diagnoses or to evaluate TH status in RTHβ patients</td>
<td>Mittag et al. 2012</td>
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<td>Acylcarnitines (ACs)</td>
<td>MS/NMR-derived metabolomics</td>
<td>In hyperthyroidism, medium-chain ACs are positively correlated with fT4; correlation with short and long-chain ACs is less well determined</td>
<td>Wong et al. 2013, Jourdan et al. 2014, Chng et al. 2016, Al-Majdoub et al. 2017, Pietzner et al. 2017, Lange et al. 2018</td>
<td></td>
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<tr>
<td>IMA</td>
<td>Single measurement</td>
<td>Increased in both hypo-and hyperthyroidism</td>
<td>Pietzner et al. 2017</td>
<td></td>
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<tr>
<td>PCs</td>
<td>MS/NMR-derived metabolomics</td>
<td>Positively correlated with fT4; research is still scarce</td>
<td>Hermenegildo et al. 2002, Arikan et al. 2007, Gù et al. 2011, Iltermann et al. 2016, Pietzner et al. 2017</td>
<td></td>
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<tr>
<td>LPS</td>
<td>MS-derived metabolomics</td>
<td>Inversely correlated with fT4; research is still scarce</td>
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<td>ADMA</td>
<td>Single measurement and MS-derived metabolomics</td>
<td>Positively correlated with fT4; also increased after adequate treatment for hyperthyroidism</td>
<td>Blanchin et al. 2003, Yu et al. 2006, Jafarzadeh et al. 2010, Pietzner et al. 2017, Alfaddeh et al. 2018</td>
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<tr>
<td>Complement proteins</td>
<td>Single measurement and proteomic analysis</td>
<td>Associations with TH were found, especially an inverse relation between fT3 and fT4 and C3; markedly influenced by other factors</td>
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<td>Phenylalanine</td>
<td>MS-derived metabolomics</td>
<td>Increased in hyperthyroidism; correlation in hypothyroidism has not been investigated yet</td>
<td>Chng et al. 2016</td>
<td></td>
</tr>
<tr>
<td>Tyrosine</td>
<td>MS/NMR-derived metabolomics</td>
<td>Increased in hyperthyroidism; correlation in hypothyroidism has not been investigated yet</td>
<td>Chng et al. 2016, Piras et al. 2017</td>
<td></td>
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<tr>
<td>Serine</td>
<td>NMR-derived metabolomics</td>
<td>Decreased in hyperthyroidism and increased in hypothyroidism; research is still scarce</td>
<td>Piras et al. 2017, 2020</td>
<td></td>
</tr>
<tr>
<td>Metabolic fingerprint</td>
<td>NMR-derived metabolomics</td>
<td>Significantly different metabolic profile in hypothyroid mice and mice with RTHβ; profile might be used to discriminate between these conditions in humans as well.</td>
<td>Boumaza et al. 2019</td>
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MS, mass-spectrometry, NMR, nuclear magnetic resonance
mice (Boumaza et al. 2019). Mass spectrometry (MS)-
derived metabolome analysis of hyperthyroid humans also
revealed altered lipids and lipid-related compounds (free
fatty acids, polyunsaturated fatty acids, acylcarnitines and
lysophospholipids) compared to healthy euthyroid controls
(Pietzner et al. 2017). A hyperthyroid state was characterized
by increased concentrations of these lipids, while LDL,
HDL and total cholesterol showed a decrease in the
hyperthyroid state as shown previously. Thus, lipogenesis
and cholesterol metabolism are highly influenced by TH.
However, markers of the lipid and cholesterol profile are
also markedly influenced by several conditions as BMI,
cardiovascular diseases, diabetes mellitus and the use of
statins. Nevertheless, the abovementioned studies found a
correlation with TH even though most of the interfering
conditions were excluded. Therefore, derivatives of lipid
and cholesterol metabolism could be used as a biomarker
to establish TH in the liver, although these markers should
be interpreted with caution in patients with cardiovascular
diseases and diabetes mellitus or using statins.

Glycerol
One of the first metabolic markers that has been
linked to thyroid status is liver glycerol-3-phosphate
dehydrogenase (GPDH) (Lee & Lardy 1965). GPDH reduces
dihydroxyacetone phosphate into glycerol 3-phosphate,
allowing the dephosphorylation of glycerol 3-phosphate
into glycerol. Hyperthyroid rats showed a significant
increase of liver GPDH indicating it is useful in measuring
intrahepatic TH status (Lee et al. 1959, Lee & Lardy 1965,
Okamura et al. 1981). It is suggested that the effect of T3
acts via coactivators and corepressors modulating GPDH
activity (Gong et al. 1998). Although GPDH could be useful
as an additional marker in assessing long-term changes
in TH status, it cannot be measured in serum (Rauchová
et al. 2011). Interestingly, Piras et al. (2021) recently found
increased glycerol concentrations in hypothyroid patients
using NMR metabolomics, which remained increased
after treatment and restoration of TH concentrations. This
suggests that liver TH status restores more slowly than
serum TH concentrations. Literature regarding glycerol as a
biomarker is scarce and more research is needed to establish
a correlation between glycerol and TH status in the liver.

CDS antigen-like (CDSL)
Recently, Nocketal. (2020) aimed to identify new biomarkers
representative for TH function by using untargeted multi-omics analyses of plasma samples from thyrotoxic
humans and mice (Engelmann et al. 2015, Pietzner et al.
2017). They showed that CDSL is a robust and promising
marker for TH status since it correlates specifically well
with serum fT3 concentrations. CDSL is a protein involved
in the modulation of leukocyte function and belongs to
the scavenger receptor cysteine-rich superfamily. Within
the liver, it is mainly expressed in macrophages (Kupffer
cells) and positively controlled by the transcription factor
LXR, which is expressed in tissues with high metabolic
activity (e.g. liver, adipocytes and macrophages) (Sanjurjo
et al. 2015). Outside the liver, the main source of CDSL is
macrophages. TH has an indirect effect on CDSL expression
which appears to be TRβ-dependent, since TRβ knock-out
mice do not show an increase in CDSL expression after
T3 exposure. Macrophages express predominantly TRα
and CDSL is thus proposed as a TRβ-dependent biomarker
reflecting hepatic TH status. Due to its TRβ dependency,
it might also be used to detect RTHβ patients. CDSL
was already suggested as a biomarker for hepatocellular
carcinoma and liver cirrhosis (Gray et al. 2009, Yamazaki
et al. 2014). It is known that CDSL is easily detectable in
serum, but literature also shows that CDSL concentrations
differ between males and females and decrease with age
(Yamazaki et al. 2014). In addition, inflammatory processes
are proved to alter this biomarker and CDSL is known to be
involved in several other diseases such as atherosclerosis,
chronic kidney disease, chronic obstructive pulmonary
disease, and cancer. In short, the study of Nock et al.
(2020) showed that CDSL is an interesting candidate as a
biomarker for hepatic TH function and it can also be used
to identify patients with RTHβ. However, it needs to be
investigated whether it is still distinctive in patients with
different types of thyroid dysfunction and comorbidities.
But since this marker could be used for both treatment and
diagnostic purposes, it seems worthwhile investigating
this biomarker.

Sex hormone-binding globulin (SHBG)
SHBG as a marker for TH action has been described
extensively (Olivo et al. 1970, Anderson 1974, Dumoulin
et al. 1995, Vierhapper et al. 1998). SHBG is a binding
protein contributing to the transportation of sex
hormones to their target cells. TH increases hepatic gene
expression of SHBG indirectly via hepatocyte nuclear
factor 4 α (HNF4α), a nuclear transcription factor, leading
to increased serum concentrations of SHBG (Pugeot et al.
1996, Selva & Hammond 2009). In hyperthyroidism, serum
SHBG concentrations are increased, whereas reduced
SHBG concentrations are observed in hypothyroidism
(Dumoulin et al. 1995, Vierhapper et al. 1998, Brenta et al.
SHBG concentrations normalize when thyroid status is restored and a significant positive correlation between SHBG and TH (fT4 and fT3) concentrations has been observed (Dumoulin et al. 1995, Hampl et al. 2003, Ito et al. 2017). Recently, SHBG concentrations in patients during treatment with a TR agonist, resmetirom, were shown to predict the effects on reduction of liver steatosis (Harrison et al. 2019). This shows the clinical applicability of this marker as a non-invasive marker of TH action. Furthermore, SHBG concentrations have been shown to discriminate between the thyrotoxic state in patients with a TSH-secreting adenoma and patients with RTH (Sarne et al. 1988, Beck-Peccoz et al. 1990). The correlation, especially in a hyperthyroid state, seems beyond question; however, there are multiple factors that interfere with SHBG concentrations as well, such as BMI, insulin resistance, age, use of oral contraceptives and pregnancy (Brenta et al. 1999, Heijboer et al. 2016). In conclusion, SHBG has been researched extensively, concentrations correlate well with serum TH concentrations and intrahepatic action and SHBG can be useful for the diagnosis of RTH and monitoring of therapy (e.g. resmetirom). However, the aforementioned interferences should be taken into account and need the appropriate attention before implementing this biomarker.

**Ferritin**

Back in the '80s, serum ferritin was proposed as a marker indicating tissue TH status (Takamatsu et al. 1985). T3 regulates hepatic ferritin expression via TRs by modulating the iron-responsive element-binding activity of the iron regulatory protein (Leedman et al. 1996). Hyperthyroid patients presented increased serum ferritin concentrations, whereas hypothyroid patients showed decreased concentrations (Kubota et al. 1993, Sachdeva et al. 2015). Ferritin concentrations decreased after treatment for hyperthyroidism and increased after treatment for hypothyroidism to concentrations within the reference interval. However, serum ferritin concentrations were not significantly correlated with TH and literature points out that between-individual variation in ferritin concentrations is large (Takamatsu et al. 1985, Fischli et al. 2017). Moreover, ferritin is strongly influenced by common circumstances such as inflammation and iron depletion. Inflammation causes increased ferritin concentrations, whereas iron depletion due to, for example, intestinal or menstrual blood loss leads to decreased ferritin concentrations. These disadvantages combined outweigh the possibilities of ferritin as a TH biomarker.

**Fibroblast growth factor 21 (FGF21)**

The hormone fibroblast growth factor 21 (FGF21) is a part of the FGF superfamily and is predominantly released by hepatocytes (Li et al. 2013). FGF21 plays a role in the regulation of glucose, lipid and energy metabolism. TH metabolism is known to affect energy metabolism as well, so TH has been linked to FGF21 expression. It was shown that T3 directly induced hepatic FGF21 expression via PPARα-dependent mechanisms (Adams et al. 2010). In addition, increased FGF21 concentrations in hyperthyroid patients and decreased concentrations in hypothyroid patients have been observed, both improving after treatment (Xiao et al. 2015, 2018, Wang et al. 2016). However, inverse correlations as well as no correlation at all are also reported (Lee et al. 2013, Bonde et al. 2014). Furthermore, mice lacking FGF21 (FGF21 knock-out mice) and WT mice displayed similar effects of TH on hepatic metabolic action indicating that TH and FGF21 most likely have independent actions on energy metabolism (Domouzoglou et al. 2014, Zhang et al. 2015). Therefore, FGF21 seems unsuitable as a biomarker reflecting hepatic TH status.

**Cardiovascular**

TH affects the heart and skeletal muscles directly by improving myocardial relaxation and indirectly by causing peripheral vasodilatation (Vargas-Uricoechea and Sierra-Torres 2014). It is known that thyroid dysfunction is strongly correlated with cardiovascular diseases (Ochs et al. 2008, Flynn et al. 2010).

**Signal peptide-CUB-EGF domain containing protein 1 (SCUBE1)**

SCUBE1 is a cell surface glycoprotein, part of the epidermal growth factor (EGF) superfamily. It is highly expressed in platelets, enhances platelet–platelet aggregation and is present in thrombi (Tu et al. 2006). The study by Erem et al. (2016) showed that patients with (subclinical) hyperthyroidism had significantly elevated concentrations of SCUBE1 compared to healthy controls. It is known that (subclinical) hyperthyroidism is associated with hypercoagulability and is, therefore, thought to increase the risk of cardiovascular diseases in this group (Erem 2011). There was no difference in SCUBE1 concentrations between subclinical and overt hyperthyroidism. SCUBE1 concentrations decreased significantly after treatment with antithyroid drugs. The authors therefore suggested that SCUBE1 could be an interesting biomarker for platelet activation and inflammation in patients with (subclinical) hyperthyroidism, especially in the earlier stages. However,
Bilir et al. (2016) showed that SCUBE1 concentrations are also increased in patients with hypothyroidism compared to healthy controls. SCUBE1 concentrations after treatment were not investigated in this study. The mechanism underlying the effect of TH on SCUBE1 concentrations has not been elucidated so far. Since both hypo- and hyperthyroidism are characterized by increased SCUBE1 concentrations, this biomarker will only be able to discriminate between a normal or abnormal thyroid tissue status and is therefore not suitable as a biomarker of tissue TH status.

Coagulation factors
Coagulation factors tissue plasminogen activator (tPA), plasminogen activator inhibitor (PAI-1) and von Willebrand factor (vWF) were associated with a hyperthyroid state (Li et al. 1998). It is hypothesized that TH influences the synthesis of endothelium-derived proteins, including tPA, PAI-1 and vWF via TRs that are expressed in endothelial cells (Dietrich et al. 1997, Burggraaf et al. 2001). Elbers et al. (2016) proved that the hypercoagulable state in hyperthyroidism is mediated through the TRβ. However, it is also hypothesized that T3 amplifies vWF secretion by affecting the adrenergic system (catecholamines acting via β2-adrenergic receptors) which regulates vWF secretion (Vischer & Wollheim 1997). Hyperthyroid patients showed significantly lower concentrations of tPA antigen compared to healthy controls and these concentrations restored in the euthyroid state. A significant inverse relationship between TH (T3 and T4) and tPA antigen concentrations was found. PAI-1 and vWF concentrations were significantly increased in hyperthyroid patients compared to euthyroid controls and decreased to normal concentrations in the euthyroid situation. Both parameters showed a significant positive correlation with TH concentrations. Conversely, hypothyroid patients can present with acquired von Willebrand's disease (VWD) with diminished vWF concentrations, which increased upon treatment (Dalton et al. 1987, Stuijver et al. 2014). This hypothyroid-associated acquired VWD is characterized by a decrease of vWF protein synthesis due to a lack of sufficient concentrations of T4 (Franchini 2004). tPA antigen and PAI-1 concentrations have not been investigated in hypothyroid patients. Proteins involved in the coagulation cascade in relation to FT4 status were also found using mass spectrometry-derived proteomics and thereby endorse the relation between TH and coagulation (Engelmann et al. 2015, Alfadda et al. 2018). Nevertheless, the abovementioned clotting factors do not seem suitable as biomarkers to adequately evaluate TH status, since the literature on these biomarkers is limited, the relation in hypothyroid patients is not clear and other conditions also affect coagulation and endothelial dysfunction, such as insulin-dependent diabetes, hemodialysis or coronary occlusions (Paramo et al. 1985, Jensen et al. 1989, Nakayama et al. 1994). However, it is important to be aware that the thyroid state influences the process of blood coagulation (Elbers et al. 2018) and that adequate treatment normalizes aberrant concentrations of its parameters.

Angiotensin-converting enzyme (ACE)
In the 1980s, a few papers were published describing the relation between angiotensin-converting enzyme (ACE) and TH. T3 is known to induce ACE protein synthesis in pulmonary artery endothelial cells, but the exact mechanism by which T3 influences ACE remains unclear (dasarathy et al. 1990). The studies showed that ACE concentrations were significantly higher in hyperthyroid patients compared to controls and significantly lower in hypothyroid patients compared to controls. Furthermore, ACE concentrations normalized after reaching euthyroidism (Smallridge et al. 1983, Graninger et al. 1986, Lee et al. 1986, Reimers et al. 1988). However, no further research has been done on this topic for the past several decades and as ACE is influenced by many factors (e.g. blood pressure regulation and salt management), we consider this biomarker as not feasible to be used in clinical practice.

Brain natriuretic peptide (BNP)
Thyroid dysfunction is associated with hemodynamic changes due to reduced heart contractility, increased cardiac output due to higher demands and even heart failure. Brain natriuretic peptide (BNP) is a marker released by the ventricular myocardium in response to volume expansion to monitor heart failure. Multiple studies showed that (NT-pro)BNP concentrations were significantly higher in hyperthyroid patients compared to hypothyroid and euthyroid patients (Schultz et al. 2004, Christ-Crain et al. 2005, Özen et al. 2007, Ertugrul et al. 2008, Ohba et al. 2020). Adequate treatment of hyperthyroidism resulted in a significant decrease of serum (NT-pro)BNP concentrations (Ertugrul et al. 2009). Furthermore, a significant positive correlation was found between (NT-pro)BNP and fT3 and fT4 concentrations and a negative correlation was found between BNP and TSH concentrations. The situation in hypothyroidism is less clear. Özen et al. (2007) and Christ-Crain et al. (2005) showed no difference in NT-proBNP concentrations between hypothyroid patients and controls, whereas Ertugrul et al. (2008) did find a
correlation. TH exerts its function on BNP via a specific TRE in the BNP gene which is activated by T3 resulting in increased BNP gene transcription (Liang et al. 2003). (NT-pro)BNP is not suitable as a functional biomarker for heart TH status since no clear relationship in hypothyroid patients was found and (NT-pro)BNP is already used as a marker to evaluate heart failure independent of TH status. However, it should be kept in mind that elevated concentrations of (NT-pro)BNP without indications for heart failure could be a sign of hyperthyroidism.

**Bone**

Bones are sensitive to TH status which is clinically emphasized by the presence of osteoporosis in hyperthyroidism. TH acts predominantly via TRα in osteoblasts. Osteoclasts and chondrocytes also express TRα1, TRα2 and TRβ1 (Bassett & Williams 2003, Williams et al. 2008, Capelo et al. 2009). Furthermore, Dio3 is expressed in osteoclasts, osteoblasts and chondrocytes, whereas Dio2 is present in mature osteoblasts making it possible to regulate TH status at the tissue level. Research in thyroidectomized postmenopausal women using L-T4 showed that biochemical euthyroidism while treated may be associated with decreased bone mineral density (Ku et al. 2021). This suggests that markers involved in bone metabolism could be of interest to determine bone TH status.

**Osteocalcin**

Osteocalcin is a carboxyglutamic acid-containing osteoblast-specific protein and is secreted in the extracellular matrix of the bone. In the carboxylated form, it plays a role in calcium binding and bone formation, whereas in the uncarboxylated form, it reaches the blood circulation and acts as a hormone by exerting an effect on multiple organs/tissues throughout the body (Karsenty & Olson 2016). The synthesis and secretion of osteocalcin are stimulated by T3 via the expression of TRs in osteoblasts (Rizzoli et al. 1986, Gouveia et al. 2001, Siddiqi et al. 2002). Serum osteocalcin concentrations are lower in hypothyroid patients (Yoneda et al. 1988, Bergmann et al. 1989, Kojima et al. 1992) and higher in hyperthyroid patients compared to euthyroid controls (Yoneda et al. 1988, Lee et al. 1990, Kojima et al. 1992, Garnero et al. 1994, Lakatos et al. 1997, Akalin et al. 2002). Osteocalcin concentrations return to normal after adequate treatment, although the study by Kojima et al. (1992) showed that normalization could take up to 24 months, while TH concentrations in the blood were already restored. A positive correlation between fT4 and fT3 concentrations and osteocalcin was observed in most studies. Other conditions such as parathyroid disorders, vitamin D deficiency and osteoporosis are known to affect this marker as well, although some studies found the correlation with TH independent of these interfering conditions. Therefore, in most cases, osteocalcin can be used as a biomarker reflecting bone TH status and thus may be useful in optimizing treatment looking at bone-related complications of thyroid disease (e.g. osteoporosis).

**Bone alkaline phosphatase**

Bone alkaline phosphatase has been considered as another marker of TH status in bone. Bone alkaline phosphatase concentrations in blood are increased in hyperthyroid patients compared to euthyroid controls (Garnero et al. 1994, Sabuncu et al. 2001, Akalin et al. 2002, Boruah et al. 2016, Ito et al. 2017) and a positive correlation was observed between bone alkaline phosphatase and fT3 and fT4 in both hypothyroid and hyperthyroid and euthyroid patients (Sabuncu et al. 2001, Boruah et al. 2016). Bone alkaline phosphatase concentrations eventually decreased after adequate treatment for hyperthyroidism, although normalization could take a few months. In contrast, hypothyroid patients did not show aberrant bone alkaline phosphatase concentrations when compared to euthyroid controls (Sabuncu et al. 2001, Boruah et al. 2016). Therefore, this biomarker is not feasible to evaluate bone TH status.

**Collagen biomarkers**

Carboxyterminal cross-linked telopeptide of type 1 collagen (ICTP) has been proposed as a marker indicating TH status as well. As previously mentioned, TH stimulates among others osteoclasts. During hyperthyroidism, osteoclasts are activated and secrete enzymes that resorb bone. In this process, fragments of collagen are released and incompletely digested. Since ICTP is a cross-link of such fragments, its concentration will increase. Several studies showed increased ICTP concentrations in hyperthyroid patients compared to euthyroid controls together with positive correlations between ICTP and fT3 and fT4 (Miyakawa et al. 1996, Loviselli et al. 1997, 2003, Nagasaka et al. 1997, Engler et al. 1999, Isaia et al. 2000). However, ICTP concentrations did not differ between hypothyroid patients and euthyroid controls (Miyakawa et al. 1996). ICTP has been proposed as a discriminative marker in distinguishing patients with TSH-secreting adenomas and RTH patients (Persani et al. 1997) and multiple guidelines recommend measuring this marker.
to discriminate between those conditions (Beck-Peccoz et al. 2013). However, the recommendation to use ICTP as a discriminative marker in patients with inappropriate secretion of TSH is based on one study describing 40 RTH patients and 10 patients with a TSH-secreting adenoma (Persani et al. 1997). Importantly, total amino-terminal peptide of procollagen type 1 (P1NP) is now commonly used as a bone marker instead of ICTP (Vasikaran et al. 2011). P1NP also correlates with thyroid status. P1NP is a precursor of collagen type 1 synthesized by osteoblasts and is considered a bone formation marker. P1NP concentrations increased in hyperthyroidism, whereas its concentrations diminished in hypothyroidism (Kužma et al. 2018, Mat Ali et al. 2020). Concentrations seem to normalize after reaching euthyroidism. However, literature regarding P1NP concentrations is still scarce, so its true relevance remains to be determined.

Tartrate-resistant acid phosphatase 5b (TRAP5b)
Tartrate-resistant acid phosphatase 5b (TRAP5b) is a specific marker of osteoclasts and reflects bone absorption (Halleen et al. 2000). Literature regarding this marker in relation to thyroid hormones is scarce and showed varying results because a decrease in TRAP5b concentrations was observed in both hyperthyroid patients compared to healthy controls and after treatment of hyperthyroidism (Jódar et al. 1997, Minisola et al. 2002). Therefore, this biomarker is not suitable to reflect bone TH status adequately.

Calcium (Ca)
Long-term exposure to T3 and T4 directly stimulates osteoclastic bone resorption (Mundy et al. 1976). Increased exposure to TH (e.g. during hyperthyroidism) thus causes an increase in serum Ca concentration (Mosekilde & Christensen 1977, Sabuncu et al. 2001, Dhanwal 2011). Ca concentrations normalized after adequate treatment (Sabuncu et al. 2001). Short-term exposure as well as subclinical hyperthyroidism and hypothyroidism did not show deviant Ca concentrations (Kobe et al. 1999, Sabuncu et al. 2001, Kiskol et al. 2003). Serum Ca is not considered as a reliable biomarker reflecting bone TH status because a hypothyroid state is not correlated with Ca concentrations and serum Ca is strongly influenced by other factors (e.g. parathyroid, intake, osteoporosis, and vitamin D deficiency).

Skeletal muscle
Creatine kinase (CK)
Several case reports described myopathy and a concordant increase in creatine kinase (CK) concentrations as the first expression of hypothyroidism (Burnett et al. 1994). Literature showed a significant increase in CK concentration in hypothyroid patients compared to euthyroid controls and a significant inverse correlation between CK and fT4 concentrations (Beyer et al. 1998, Hekimsoy & Kavalali Oktem 2005, Hong et al. 2021). After treatment for hypothyroidism, CK concentrations normalized (Klein et al. 1980, Khaleeli & Edwards 1984, Nakahama et al. 2001, Hekimsoy & Kavalali Oktem 2005). In hyperthyroidism, CK concentrations seemed to decrease, but literature is scarce (Wan Nazaimoon et al. 2001). Increased CK concentrations were also found in hyperthyroid patients receiving antithyroid therapy indicating a short period of hypothyroidism when therapy is initiated (Suzuki et al. 1997, Cheng et al. 2020). The mechanism remains unclear; it is unlikely that TH directly affects CK concentrations since high concentrations of T4 and T3 did not reduce CK concentrations in vitro (Smith 1976). Knowledge on CK and hyperthyroidism is limited, but CK concentrations might be useful to get an impression of TH status in skeletal muscle during hypothyroidism. However, it should be kept in mind that CK indicates cell degradation which is also seen in, for example, myocardial infarction and therefore lessens its specificity as a marker of TH status in skeletal muscle.

Intestine
TH exerts its effect on the intestine via the epithelial cells, the enterocytes (Ebert 2010). T3 affects the development of the intestinal epithelium and regulates the levels of specific brush-border enzymes via TRα1 (Plateroti et al. 2001). There is increasing evidence of the existence of a thyroid–gut axis (Knezevic et al. 2020), highlighting the connection between these two organs.

Intestinal alkaline phosphatase (IAP)
Intestinal alkaline phosphatase (IAP) is expressed and secreted by intestinal epithelial cells and its expression is highest in the duodenum. IAP’s primary function is to maintain intestinal homeostasis. T3 increased the transcription of IAP in both humans and rats (Hodin et al. 1992, 1996, Malo et al. 2004) and serum IAP concentrations were decreased in hypothyroid rats (Watson & Tuckerman 1971, Hodin et al. 1996, Malo et al. 2004). However, IAP concentration as a biomarker of TH status in the intestine has not been further investigated. It might be an interesting biomarker to evaluate intestinal TH status and gain more insight into intestinal symptoms in thyroid disorders, which could lead to better treatment.
Kidney

Another TH target organ is the kidney, which expresses DIO1. TH regulates the expression of ion channels and transporters via TRα1 directly (Li et al. 2002). Indirectly, decreased renal blood flow in hypothyroidism (due to decreased cardiac output, myocardial contractility and increased peripheral resistance) can cause an increase in creatinine and thus decrease of estimated glomerular filtration rate (eGFR) (Chou et al. 2011).

Creatinine

Hypothyroidism is in a large part of the patients characterized by a reversible reduction of GFR, whereas hyperthyroidism causes an elevation in GFR (Chou et al. 2011). This results in an elevation of the creatinine concentration in hypothyroidism and a reduction of creatinine concentration in hyperthyroidism (Aizawa et al. 1986, Ford et al. 1989, Lafayette et al. 1994, Shirota et al. 1992, Verhelst et al. 1997). This pattern is confirmed by NMR-metabolomic studies (Piras et al. 2017, 2021). Since creatinine is a general readout of kidney function, it cannot be used as a TH-specific biomarker.

Pancreas

Homeostasis model assessment for insulin resistance (HOMA-IR)

TH has direct effects on insulin production as T3 and TRα stimulate the proliferation of pancreatic islet cells and T3 is necessary for the transition of islets to glucoreponsive insulin-secreting cells (Furuya et al. 2010). Homeostasis model assessment for insulin resistance (HOMA-IR) is a model to assess insulin resistance based on fasting insulin and fasting glucose and has widely been investigated in relation to thyroid hormones and thyroid function. Literature reported both a negative association between HOMA-IR and both fT4 and fT3 in euthyroid participants (Roos et al. 2007, Abdelwhab & Foda 2010, Bin Obead et al. 2021), a positive association (Ren et al. 2014, Wang et al. 2018) as no association at all (De Pergola et al. 2007, Kazuakaussiene et al. 2021). In both hypothyroid and hyperthyroid patients, increased levels of HOMA-IR were found compared to euthyroid controls (Kapadia et al. 2012, Srivastava et al. 2021), although no difference between hypothyroid patients and euthyroid controls was found as well (Owecki et al. 2006). In conclusion, HOMA-IR is a model assessing insulin resistance with possible associations with thyroid function but should not be used as a TH tissue biomarker.

Non-organ specific markers

Trace elements

Copper (Cu) and selenium (Se) An increase in T3 and T4 is associated with an increase in Cu concentrations (Mittag et al. 2012, Blasig et al. 2016). Cu is a trace element and important for growth and development. Cu deficiency leads to anemia, growth defects, and susceptibility to infections. Research in mice showed that the liver is an important organ in Cu homeostasis which is affected by TH via the TRβ, the main TR in the liver (Flores-Morales et al. 2002, Cheng et al. 2010). TH downregulates competing intracellular Cu-binding proteins and enhances synthesis and export of hepatic ceruloplasmin. 95% of Cu in healthy human plasma is carried by the transport protein ceruloplasmin (Hellman & Gitlin 2002). T3 provides an increased expression of transporters ATP7A and ATP7B and ceruloplasmin and decreases the production of Cu-containing enzymes SOD1, Mtt1 and Mtt2 (Mittag et al. 2012). It is conceivable that ceruloplasmin might be correlated to TH, but no research has been performed yet regarding ceruloplasmin as a potential interesting biomarker. Blasig et al. (2016) showed that Cu concentrations were positively correlated with plasma T3 and T4 concentrations in children with congenital hypothyroidism and suggested that Cu status could serve as a diagnostic tool for monitoring the effect of TH substitution in these children or even at a more population-based scale. The study of Jain (2014) confirmed this correlation in the overall population for fT4 and TT4 in men and TT3 and TT4 in women. However, despite the fact that Cu concentrations are very stable and not susceptible to small changes, Cu concentrations are about 20% higher in males compared to females (Jain 2014) and other elements as cadmium can influence their concentrations. Furthermore, it should be taken into account that Cu is an acute phase reactant and its concentration increases during inflammation. Concluding, Cu does not contribute as a biomarker reflecting tissue TH status.

Another trace element that has been positively correlated to T3 concentrations is Se (Mittag et al. 2010, 2012). Se is required for the production of selenoproteins (DIO1, DIO2 and DIO3). Decreased Se concentrations together with decreased serum TH concentrations were observed in critical illness (Gärtner 2009). However, after recovery, T3 and T4 concentrations improved to normal, whereas Se concentrations remained low (Angstwurm et al. 2004). Furthermore, not all studies showed a positive correlation between Se and TH (Kralk et al. 1996, Kucharzewski et al. 2003, Blasig et al. 2016, Rasic-Milutinovic et al. 2017) and Se...
concentrations are influenced by inflammation. Therefore, its relevance as a TH biomarker is doubted.

The combination of Cu and Se has been studied to discriminate between RTH and TSH-secreting pituitary adenoma (Mittag et al. 2012). RTH is characterized by high serum fT3 and fT4 and normal/slightly elevated TSH and TSH-secreting pituitary adenoma can be presented with the same biochemical abnormalities. Based on decreasing Cu and increasing Se concentrations observed in RTH patients compared to healthy controls, the authors concluded that the Se/Cu ratio might be useful as a biomarker, since the Se/Cu ratio increased in RTH patients compared to patients with TSH-secreting pituitary adenoma. Furthermore, the Se/Cu ratio could be used to assess TH therapy in RTH patients. Cu concentrations increased after initiation of therapy due to TR activation, whereas Se concentrations remained stable, indicative of TRβ resistance (Moran et al. 2013). A decreased Se/Cu ratio could then indicate therapy response. Cu and Se concentrations are easily measured in serum and therefore potentially feasible. However, as mentioned before, both Cu and Se are influenced by other factors. To conclude, the Se/Cu ratio is potentially interesting as a biomarker in the diagnosis of RTH; however, its role should be handled with caution due to possible interferences.

**Fatty acid metabolism**

**Acylcarnitines (ACs)** Acylcarnitines (ACs) play an important role in the transport of long-chain fatty acids (LCFA) across the mitochondrial membrane. LCFA are acylated, resulting in the formation of acyl-coenzyme A (CoA). Carnitines interact with acyl-CoA under influence of carnitine palmitoyltransferase 1 (CPT-1) on the outer aspect of the inner mitochondrial membrane to form free CoA and acylcarnitine. ACs are transported across the inner mitochondrial membrane in exchange for carnitine. In the inner membrane, the acyl group is re-esterified with CoA by CPT2, resulting in acyl-CoA and carnitine. Acyl-CoA is converted into acetyl-CoA, which will go into the tricarboxylic acid cycle. It is known that TH affects β-oxidation and oxidative phosphorylation. T3 increases the expression of the CPT1 gene via the TRα resulting in elevated CPT1 concentrations, which enhances fatty acid β-oxidation (Mullur et al. 2014). The AC profile consists of short, medium and long-chain ACs. Short-chain ACs originate from glucose and amino acid metabolism, whereas medium and long-chain ACs are derived from fatty acid metabolism (Al-Majdoub et al. 2017). Many studies using metabolomics showed a correlation between TH status and ACs. In healthy men supplemented with L-T4 to mimic a hyperthyroid state, medium-chain ACs increased at the cost of short-chain ACs (Pietzner et al. 2017). Furthermore, hyperthyroid patients receiving treatment showed reduced concentrations of medium-chain, long-chain and total ACs after reaching euthyroidism in one study (Ching et al. 2016), whereas the study of Al-Majdoub et al. (2017) only found this reduction in medium-chain ACs. Short-chain ACs were elevated and long-chain ACs were unchanged. In euthyroid participants, positive associations between fT4 and ACs were found; however, no distinction was made between differences in chain length (Jourdan et al. 2014, Lange et al. 2018). In contrast, Wong et al. (2013) investigated AC profiles related to hypothyroidism, euthyroidism and hyperthyroidism and found no difference in these concentrations between the three different thyroid states. It is possible that due to their small sample size (six hypothyroid and six hyperthyroid patients) and lack of a control group (the euthyroid groups consisted of the treated hypo- and hyperthyroid group), outcomes are deviant. Overall, there is a correlation between ACs and fT4 concentrations, which indicates this profile could be of interest as a potential biomarker for establishing TH status. However, there are some discrepancies between the several studies. Previous literature showed that medium-chain ACs are quickly lowered after refeeding in the fasting state, while long-chain ACs remained stable (Ramos-Roman et al. 2012). Since the transition from hyperthyroidism to euthyroidism is characterized by decreased metabolic demand, this could explain that predominantly medium-chain ACs were altered and long-chain ACs remained stable. However, outcomes regarding the correlation of short and long-chain ACs differed between the studies. Most studies sampled blood in the morning in the fasting state, so this could not have affected the outcomes. It might be due to dietary habits (Kien et al. 2014). It is known that diet influences AC metabolism and since populations from different continents with different dietary habits were included, this could explain the altered results concerning chain length. Since multiple studies using metabolomics found a significant positive relationship between ACs and fT4 concentrations and especially medium-chain ACs were altered, ACs have potential as a biomarker reflecting TH status. They could be helpful in giving a better representation of euthyroidism and optimizing the treatment of thyroid dysfunction. Future studies should focus on evaluating the relevance of the different AC chain lengths in relation to TH status. Furthermore, research needs to prove whether TH can be related to one specific AC or a profile of (e.g. medium-chain) ACs would correlate better.
Oxidative stress

Hyperthyroidism and subclinical hyperthyroidism are associated with oxidative stress and, therefore, with elevated markers of lipid peroxidation. Free radicals, a result of oxidative stress, are able to react with free fatty acids, the major energy source of thyrocytes, resulting in lipid peroxidation. Malondialdehyde (MDA) is an important lipid peroxidation marker, which is produced in response to cell death in peripheral tissues. Significantly elevated concentrations of MDA were observed in patients with (subclinical) hyperthyroidism compared to euthyroid controls (Adali et al. 1999, Alicigüzel et al. 2001, Cetinkaya et al. 2005, Erdamar et al. 2008, Erem et al. 2015) and in patients with hyperthyroidism compared to subclinical hyperthyroidism (Erem et al. 2015). Alicigüzel et al. (2001) found a significantly positive correlation between MDA and serum T3 and T4 concentrations. Treatment of (subclinical) hyperthyroidism resulted in significantly decreased plasma MDA concentrations, although normalization was not reached in every study (Adali et al. 1999, Erdamar et al. 2008, Erem et al. 2015). Two studies investigated both hyper- and hypothyroidism and showed increased MDA concentrations in hypothyroid patients compared to controls as well (Erdamar et al. 2008, Lassoued et al. 2010).

Ischemia-modified albumin (IMA) is a modified form of albumin due to oxidative damage and has first been reported as a marker for cardiac ischemia. However, IMA turned out to be elevated in non-cardiac diseases as well. A recent meta-analysis of 12 studies showed a significant increase in IMA concentrations in hyperthyroid patients compared to euthyroid controls. The same was observed in hypothyroid patients and no significant difference in IMA concentrations was found before and after treatment. IMA levels correlated negatively with T4, fT4 and fT3 concentrations in hyperthyroid patients and positively with fT4 and fT3 concentrations in hyperthyroid patients (Reddy et al. 2017). Since both hyper- and hypothyroidism resulted in increased MDA and IMA concentrations, we can conclude that these biomarkers are not specific to act as a marker for tissue TH status. However, oxidative stress remains associated with hyperthyroidism, which is also demonstrated by (non-targeted) metabolome and proteome analysis, revealing several biomarkers indicating augmented defense against oxidative stress (Jourdan et al. 2014, Al-Majdoub et al. 2017, Pietzner et al. 2017, Lange et al. 2018). Concentrations of γ-glutamyl amino acids (GGAA) were positively correlated with fT4 concentrations, whereas phosphatidylcholines (PCs) and lysophospholipids (LPs) showed a negative correlation with fT4 concentrations. GGAA is part of the γ-glutamyl cycle (GGC), a pathway for metabolizing glutathione and plays a role in detoxification. TH is thought to stimulate the GGC in its antioxidant effect. Furthermore, research in rats showed that T3 induces the hepatic activation of a transcriptional factor (Nrf2) of the major cellular defense mechanism against oxidative stress (Romanque et al. 2011, Cornejo et al. 2013). Decreased PCs and LPs together with increased fT4 concentrations are most likely the result of enhanced lipid oxidation, which strengthens the relation of TH with oxidative stress. Although several biomarkers indicate that (defense against) oxidative stress seems to be related to TH, research is still too scarce to conclude these are all adequate biomarkers determining TH status.

Asymmetric dimethylarginine (ADMA)

Untargeted mass-spectrometry proteomics revealed a positive correlation of fT4 with asymmetric dimethylarginine (ADMA) (Pietzner et al. 2017). ADMA is produced as a by-product of systemic proteolysis, which is triggered by TH excess (Müller & Seitz 1984). It favors hypertension by directly impairing nitric oxide (NO)-dependent vasodilatation and, therefore, seems to increase the risk of cardiovascular diseases. This correlation with thyroid function is supported by previous literature (Hermenegildo et al. 2002, Arikan et al. 2007, Gu et al. 2011, Ittermann et al. 2016). However, ADMA concentrations were also elevated in well-treated patients with former hyperthyroidism and hypothyroidism, which diminishes its clinical application as a marker for TH status.

Complement proteins

The complement system is an important component of innate immunity. Complement proteins are predominantly produced by B-lymphocytes in response to infectious and inflammatory processes. However, also thyrocytes are able to produce complement proteins, which suggests a relation between complement proteins and thyroid hormones (Blanchin et al. 2003). Proteomic analyses revealed associations between thyroid state and several proteins of the complement system (Pietzner et al. 2017, Alfadda et al. 2018), namely complement factor H-related protein 5, factor H and complement factor C2, C3 and C4a. This association has been proposed in previous literature (Yu et al. 2006, Jafarzadeh et al. 2010) and specifically the inverse association between fT3 and fT4 with C3 was confirmed. However, other conditions (e.g. inflammation, multiple autoimmune diseases) increase the concentration of different complement proteins and the complement system compasses a broad range of proteins, which
complicates the selection of proteins associated with TH. Therefore, more literature is needed to establish the value of complement proteins in determining TH status and they can currently not serve as feasible biomarkers evaluating TH status.

**Amino acids**

A variety of studies using (un)targeted metabolomics found multiple alterations in several amino acid concentrations depending on TH status. Tyrosine concentrations were elevated in hyperthyroid patients compared to euthyroid controls, as well as phenylalanine, a precursor of tyrosine (Chng et al. 2016, Piras et al. 2017). During the synthesis of TH, iodination of tyrosyl residues occurs, which might be a reason that increased TH production in hyperthyroid patients resulted in elevated concentrations of tyrosine and phenylalanine. Serine concentrations were decreased during hyperthyroidism and increased during hypothyroidism and remained increased after adequate treatment of hypothyroidism (Piras et al. 2017, 2021). The decrease in serine concentrations is thought to be explained by increased uptake of serine for gluconeogenesis purposes in hyperthyroid patients while hypothyroidism is characterized by a hypometabolic state explaining the increased concentrations of serine.

**Metabolic fingerprint**

Another approach to look at this massive amount of data is using profiles to distinguish between different conditions. Boumaza et al. (2019) used untargeted NMR-based metabolomics to discriminate mice with resistance to TRα (RTHα), due to mutations created in the THRA gene encoding for TRα, from hypothyroid mice and control mice. The study showed no specific biomarker to be discriminative for RTHα, hypothyroidism and euthyroidism in mice but the metabolic fingerprint of the RTHα mice was significantly different compared to control mice and ‘normal’ hypothyroid mice. In distinguishing RTHα and hypothyroid mice, especially differences in the lipid and cholesterol metabolism were detected. Therefore, using the whole metabolic profile, metabolomics might also be useful in humans to diagnose RTHα more easily and distinguish this rare disease from ‘normal’ hypothyroidism. It should be further elucidated how cut-off values for these metabolic fingerprints are shaped. However, the approach of discriminating between conditions upon profiles using OMICs has already been performed in humans and might be promising and worthwhile to investigate more extensively for this purpose (Friedrich et al. 2020).

**Conclusion and recommendations**

This review addressed the need for new biomarkers in determining tissue TH status. We summarized several biomarkers that, in the current literature, have been related to TH status in a variety of tissues and systems and can contribute to a diagnosis. Most of the biomarkers discussed are by themselves not considered suitable to serve as adequate biomarkers to indicate TH status. However, a few of them are promising (Fig. 2).

Derivatives of lipid and cholesterol metabolism could be used as biomarkers to establish TH status in the liver, although appropriate attention should be paid to conditions that influence these pathways. Glycerol is proposed as a biomarker to represent liver TH status in thyroid disorders. GPDH showed a strong correlation with TH status but cannot be measured in blood. Glycerol, which can be measured in blood, is a substrate of GPDH and could therefore be an attractive alternative (Piras et al. 2021). Recently, CDSL has been proposed as a TRβ-dependent biomarker for hepatic TH status although only in the hyperthyroid state (Nock et al. 2020). A few limitations of this marker have been discussed previously. Notably, inflammatory processes are suspected to influence this marker; it might therefore be interesting to assess whether CDSL could still function as a reflection of hepatic TH status in autoimmune thyroid diseases. SHBG can be used as a non-invasive hepatic biomarker to monitor the treatment outcome of TH therapy in patients with NASH (Harrison et al. 2019). Furthermore, osteocalcin and P1NP might be interesting indicators of bone TH status. Available research regarding IAP as a biomarker of intestinal TH status seems promising and the biomarker can be measured in serum via electrophoresis. Although not organ-specific, the Se/Cu ratio could serve as a biomarker to evaluate treatment effect in RTHα and RTHβ patients, since TSH is not always the correct indicator for a sufficient TH concentration in all tissues (Mittag et al. 2012). Several studies using metabolomics found a positive association between ACs and TH status in the hyperthyroid state making an AC profile or specific ACs of interest as an overall marker indicating tissue TH status (Jourdan et al. 2014, Chng et al. 2016, Al-Majdoub et al. 2017, Pietzner et al. 2017, Lange et al. 2018).

Biomarkers can also be useful in clinical practice to discriminate between thyroid disorders. CDSL might be useful in diagnosing patients with RTHβ, due to its TRβ-dependent action. SHBG has been proposed as a useful tool to discriminate between RTH and TSH-secreting adenoma (Sarne et al. 1988, Beck-Peccoz et al. 1990). Cu and Se were
mentioned as predictors of TH status but have never really gained a foothold as official biomarkers. However, the Se/Cu ratio also seems promising in order to differentiate between RTHβ and TSH-secreting pituitary adenoma (Mittag et al. 2012). Another interesting approach was the use of a metabolic profile to distinguish between RTHα and hypothyroidism in the diagnostic process instead of a single biomarker, although this discriminative effect still has to be confirmed in humans (Boumaza et al. 2019).

The goal of this review was to assess potential tissue TH biomarkers in serum, since it is not feasible in humans to assess tissue TH status using a biopsy. In this review, most studies described the correlation between the proposed biomarker and TH status based on a hypo- or hyperthyroid state compared to a (treated) euthyroid state. Certainly, the response of the proposed biomarker upon TH fluctuation is important in exploring a possible correlation, although it does not necessarily prove that the biomarker indicates an altered tissue TH status in a patient who is biochemically euthyroid. Studies that investigate tissue TH biomarkers in patients before and after thyroidectomy due to thyroid carcinoma, and thus comparing a euthyroid state without and without L-T4, might be better to detect alterations at the tissue level when serum TH is considered euthyroid. Hitherto, the few studies that investigated this specific patient group confirmed the proposed correlation with cholesterol and bone biomarkers. Therefore, future research in thyroidectomized patients after thyroid carcinoma might be of interest to detect other promising biomarkers.

Currently, not only OMIC techniques are used to map tissue TH status, but also a TH action indicator mouse (THAI) model which is an interesting and alternative way to study the tissue TH status (Mohácsik et al. 2018). This animal model visualizes TH signaling in vivo via luciferase activity, allowing many different tissues to be examined under a variety of experimental conditions. Although this model is not relevant for daily clinical practice, it illustrates that there is a need for knowledge about tissue TH status in relation to systemic TH concentrations.

In summary, a variety of old and new biomarkers are available for establishing tissue TH status and using these
for treatment and diagnostic purposes. The proposed biomarkers of interest all have in common that further exploration is needed to determine their value as a tissue-specific biomarker for TH status. Recently described and of potential interest are ACs. In the future, a thyroid panel combining several specific biomarkers fully capturing thyroid status in serum and most target tissues might be worthwhile investigating. To achieve that goal, more research is first needed to validate the aforementioned biomarkers of interest, followed by the composition of such a thyroid panel using these biomarkers.

Declaration of interest

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