

Mechanistic analysis of metabolomics patterns in rat plasma during administration of direct thyroid hormone synthesis inhibitors or compounds increasing thyroid hormone clearance



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HIGHLIGHTS

- Male and female rats treated with reference compounds producing thyroid toxicity.
- Patterns of common metabolite changes of thyroid effects were established.
- Metabolites separating indirect and direct thyroid effects were identified.
- Tox patterns for direct and indirect thyroid effects are different in male and female rats.
- Biochemical explanation of MoA relevant metabolites is provided.

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ABSTRACT

For identification of toxicological modes of action (MoAs) a database (MetaMap®Tox) was established containing plasma metabolome consisting of approximately 300 endogenous metabolites. Each five male and female Wistar rats per groups were treated with >500 reference compounds over a period of 28 days. More than 120 specific toxicity patterns of common metabolite changes associated with unique MoAs were established.

To establish patterns predictive effects on the thyroid, animals have been treated with reference compounds directly acting on the thyroid hormone formation (such as methimazole, ethylenethiourea) as well as liver enzyme inducers leading to an increased excretion of thyroid hormones and therewith to a secondary response of the thyroid (such as aroclor 1254 and boscalid). Here we present the plasma metabolite changes which form the patterns for direct and indirect effects on the thyroid. It is possible to identify metabolites which are commonly regulated irrespective of an indirect or direct effect on the thyroid as well as groups of metabolites separating both MoAs. By putting the metabolite regulations in the context of affected pathways helps to identify thyroid hormone inhibiting MoAs even when the hormone levels are not consistently changed. E.g., direct thyroid hormone synthesis inhibitors affect some enzymes in the urea cycle, increase the ω -oxidation of fatty acids and decrease glutamate and oxoproline levels, whereas indirect thyroid hormone inhibiting compounds interact with the lipid mediated and liver metabolism.

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1. Introduction

BASF SE and metanomics GmbH have established characteristic profiles of endogenous metabolites in rat plasma correlated to toxicological effects of multiple modes of action (MoAs) with a single repeated dose study. For that purpose, a comprehensive database (MetaMap®Tox) has been built up. About 300 metabolites are

measured in plasma samples of Wistar rats after 4 weeks repeated administration of about 500 pharmaceutical, chemical and agrochemical compounds for which the toxicity profile is well known. Sets of common metabolite level changes (metabolite patterns) were arranged to characterize their toxicological MoAs.

The thyroid gland is one of the largest of the endocrine tissues. Most thyroid-toxicological events are associated with the follicular cells that are responsible for synthesis, storage and secretion of the thyroid hormones thyroxine (T₄) and 3,5,3'-triiodothyronine (T₃).

The hypothalamus secretes thyrotrophin releasing hormone (TRH). This hormone stimulates the thyrotropic cells within the

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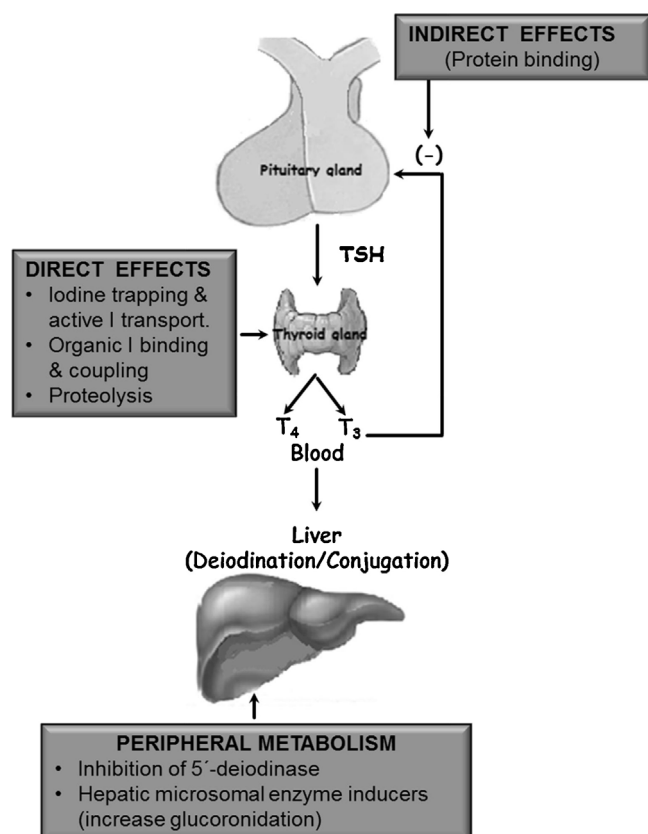


Fig. 1. Schematic representation of the possible sites of interaction of compounds that causes hypothyroidism.

Adapted from Davies (1993).

pituitary gland to release thyroid stimulating hormone (TSH). The activity of the thyroid follicular cells is regulated by the concentration of TSH, acting on the thyroid gland to stimulate the synthesis and release of T₃ and T₄. In the circulation, both are protein bound (in humans to a high-affinity protein called thyroxine binding globuline (TBG) and in rodents to a nonspecific transport and low affinity protein, albumin (prealbumin and postalbumin), Dohler et al., 1979) and less than 1% of these hormones circulate as free-T₃ and free-T₄ (Davies, 1993). The inhibitory effects of thyroid hormones and the stimulatory action of TRH (via the hypothalamic–hypophyseal portal system) regulate TSH production in order to maintain optimal thyroid hormone levels in plasma and reflect a feed-back control interrelationship between the secretion and plasma concentration of the thyroid hormones, on one hand, and the secretion of thyrotropin releasing hormone, on the other.

It is worth noting that only free hormones are physiologically active and T₃, mainly produced by the deiodination of T₄, is about four fold more biologically active on target tissue nuclear receptors than T₄. The liver conjugates T₃ and T₄ by a process of glucuronidation and sulphation, promoting their excretion via bile into the small intestine (Coelho-Palermo and van Ravenzwaay B., 2007; Cunha and van Ravenzwaay B., 2005; Davies, 1993). Thus, concentrations of circulating thyroid hormones depend not only on the rate of secretion from the thyroid gland but also on how fast the hormones are cleared.

Several compounds induce hypothyroidism by different MoAs, these are illustrated in Fig. 1. Despite these differences in toxicological MoAs, their consequences are quite similar. The most common change is thyroid follicular cell hyperplasia and hypertrophy mediated by an increased release of TSH from the anterior pituitary in

response to reduced circulating levels of thyroid hormone. There is also evidence that sustained thyroid follicular-cell hypertrophy and hyperplasia can lead to tumor formation in rats (Hood et al., 1999; Klaassen and Hood, 2001; Meek et al., 2003). Additionally, it has been suggested that induction of T₃ glucuronidation, rather than T₄ glucuronidation, mediates increases in serum TSH of rats treated with microsomal enzyme inducers (Klaassen and Hood, 2001; McClain, 1989). By contrast, thioamides such as methimazole and 6-propyl-2-thiouracil (PTU) inhibit the enzyme thyroid peroxidase (TPO) in the thyroid, reducing the synthesis of T₃ and T₄, thereby blocking uptake of iodotyrosines from the colloid. They also block iodine release from peripheral hormones (Braverman and Cooper, 2012).

In general, direct mechanisms on thyroid hormone synthesis comprise the inhibition of iodide uptake by the thyroid gland, organification defects from inhibiting TPO catalyzed reactions, the inhibition of thyroid hormone secretion through an excess of iodide and thyroid cytotoxicity. Otherwise, indirect mechanisms are the inhibition of TSH synthesis, competition for thyroid hormone binding proteins or inhibition of T₄ deiodination and changes in thyroid hormone conjugation (such as the induction of UDP-glucuronosyltransferase).

Distinguishing between direct and indirect effects in the thyroid is of great significance for the regulatory assessment of thyroid-related findings. To date, the perchlorate discharge assay has been used for this purpose (Coelho-Palermo and van Ravenzwaay B., 2007). Within the framework of metabolomics, compounds known to induce thyroid toxicity through direct and indirect mechanisms were investigated for their common profile changes. For the first case the pattern is referred to as the “thyroid toxicity direct pattern” the reference compounds were ethylenethiourea (ETU), 6-propyl-2-thiouracil (PTU) and methimazole. For the “thyroid toxicity indirect pattern” the selected reference compounds were pendimethalin, fipronil, boscalid and aroclor 1254.

The aim of this publication is the mechanistic analysis of the regulation of metabolites in the aforementioned patterns.

2. Materials and methods

2.1. Animals and maintenance conditions

Wistar rats (CrI:WI(Han)) were supplied by Charles River, Germany at an age of 59–67 days at the beginning of the studies and underwent an acclimatization period of 1 week. The animals were singly housed in standard cages (floor area 800 cm²), supplied by Becker & Co., Castrop-Rauxel, Germany. The animals were maintained in an air-conditioned room at a temperature of 20–24 °C, a relative humidity of 30–70%, and a 12 h light/12 h dark cycle. Before the animals' arrival, the room was completely disinfected using a disinfectant (“AUTEX”, fully automatic, formalin-ammonia-based terminal disinfectant, supplied by Dr. Gruf KG, Neuss, Germany). During the study, the floor and walls were cleaned weekly with a solution of 0.1% Incidin® (supplied by Henkel, Düsseldorf, Germany) in water. Ground Kliba mouse/rat maintenance diet was supplied by Provimi Kliba SA, Kaiseraugst, Switzerland. The diet and drinking water were available *ad libitum* (except immediately before blood sampling) and regularly assayed for chemical contaminants and the presence of microorganisms.

2.2. Treatment of animals with compounds

The studies were performed according to the German Animal Welfare legislation 23 177-07/G 08-3-001. The laboratory is AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) certified.

The compounds were administered via either the feed or via gavage to each group (five rats per sex) in individual tests and the doses were chosen based on BASF internal studies or literature data and reflected the 28 days maximum-tolerated dose (MTD) (Table 1).

2.3. Blood sampling

Between 7:30 and 10:30 h, blood samples were taken from the retrobulbar sinus in all rats under isoflurane anesthesia (1.0 ml K-EDTA blood on study days 7, 14 and 28) after a fasting period of 16–20 h. The blood samples were centrifuged (10 °C, 2000 × g, 10 min) and EDTA plasma was separated. The EDTA plasma

Table 1
Dose levels and route of administration of test compounds.

Test compound	Dose level	Route of administration	MoA
Methimazole	100 mg/kg body weight	Gavage (water)	Direct thyroid toxicity
6-Propyl-2-thiouracil (PTU)	10 mg/kg body weight	Gavage (water)	Direct thyroid toxicity
Ethylenethiourea (ETU)	300 ppm	Food	Direct thyroid toxicity
Boscalid	15000 ppm	Food	Indirect thyroid toxicity
Metazachlor	20000 ppm	Food	Indirect thyroid toxicity
Aroclor 1254	25 mg/kg body weight	Gavage (corn oil)	Indirect thyroid toxicity
Fipronil	300 ppm	Food	Indirect thyroid toxicity
Pendimethalin	10000 ppm	Food	Indirect thyroid toxicity
L-Thyroxine	0.5 mg/kg body weight	Gavage (water)	Thyroid hormone

samples were covered with nitrogen and frozen at -80°C until metabolite profiling was performed.

2.4. Metabolite profiling

For mass spectrometry-based metabolite profiling analysis, K-EDTA plasma samples taken on study days 7, 14 and 28 were extracted by a proprietary method. Three types of mass spectrometry analysis were applied to all samples: GC-MS (gas chromatography–mass spectrometry) and LC-MS/MS (liquid chromatography–tandem mass spectrometry) were used for broad profiling, as described in (van Ravenzwaay B. et al., 2007). Proteins were removed from plasma samples by precipitation. Subsequently polar and non-polar fractions were separated for both GC-MS and LC-MS/MS analysis by adding water and a mixture of ethanol and dichloromethane. For GC-MS analysis, the non-polar fraction was treated with methanol under acidic conditions to yield the fatty acid methyl esters derived from both free fatty acids and hydrolyzed complex lipids. The non-polar and polar fractions were further derivatized with O-methyl-hydroxylamine hydrochloride and pyridine to convert oxo-groups to O-methyl-oximes and subsequently with a silylating agent before analysis (Roessner et al., 2000).

For LC-MS analysis, both fractions were reconstituted in appropriate solvent mixtures. HPLC was performed by gradient elution using methanol/water/formic acid on reversed phase separation columns. Mass spectrometric detection technology was applied which allows target and high sensitivity MRM (Multiple Reaction Monitoring) profiling in parallel to a full screen analysis (Walk and Dostler, 2003).

Steroids hormones, catecholamines and their metabolites were measured by online SPE-LC-MS/MS (Solid phase extraction-LC-MS/MS) (Yamada et al., 2002). The methodological process is represented in Fig. 2.

Data were normalized to the median of reference samples which were derived from a pool formed from aliquots of all samples to account for inter- and intra-instrumental variation.

2.5. Further examinations

All animals were checked daily for any clinically abnormal signs and mortalities. Food consumption was determined on study days 7, 14, 21 and 28. Body weight was determined before the start of the administration period in order to randomize the animals and on study days 0, 4, 7, 14, 21 and 28.

At the end of the treatment period, the animals were sacrificed by decapitation under Isoflurane anesthesia.

2.6. Statistics

2.6.1. Metabolite profiling

The data were analyzed by univariate and multivariate statistical methods. The sex- and day-stratified heteroscedastic *t*-test (welch test) was applied to log-transformed quantitative and semi-quantitative metabolite data to compare treated groups with respective controls. *p*-Values, *t*-values, and ratios of corresponding group medians were collected as metabolic profiles and fed into a database (MetaMap® Tox). Metabolic patterns presented were developed applying 5% significance level and demanding statistical significance during at least two out of three (study days 7, 14 and 28) time points.

2.6.2. Pair-wise comparison

The pair-wise comparisons (PWCs) were conducted by calculating Welch *t*-values from treatment and control samples of each metabolite stratified by sex and time. With the pair-wise comparison the profiles (*t*-values) of an unknown compound were compared with each profile of the compounds in the MetaMap® Tox database. These comparisons were quantified by calculating either the parametric Pearson product moment correlation coefficient or the non-parametric Spearman rank correlation coefficient. The similarity between the profiles of the unknown compound and the compounds of the MetaMap® Tox database was ranked by the resulting correlation coefficients in descending order. A discrepancy between the Pearson and Spearman coefficient was often due to some greatly changed metabolite levels strongly affecting the Pearson, but not the Spearman correlation coefficient, the latter being more reliable in this case. A coefficient of about >0.500 for male

animals, and >0.600 for female animals indicated a good correlation based on the distribution of all coefficients calculated from the MetaMap®Tox database (Mattes et al., 2013).

3. Results and discussion

As mentioned in Section 2, the doses of the compounds were chosen based on the 28 days MTD. Changes in body weight and food consumption as rough measure of general toxicity noted upon administration of the test compounds are shown in Table 2. Body weight decreases $>10\%$ were noted for methimazole, ETU, PTU, metazachlor and pendimethalin. The thyroid effect reference compounds are well-known and therefore, there is certainty that the administered doses, elicited the desired mode of action.

Sets of common metabolite level changes (metabolite patterns) were arranged to characterize thyroid toxicological MoAs. Following the administration of the aforementioned compounds (see Table 1) known to induce hypothyroidism, metabolite patterns were established to differentiate between a direct effect (thyroperoxidase inhibition) and an indirect effect (increased thyroid hormone conjugation via induction of liver microsomal enzymes) on thyroid hormone homeostasis. After identification of the significantly changed metabolites and a consistency check through an expert panel, the patterns were validated against the MetaMap®Tox data base (van Ravenzwaay B. et al., 2010). Afterwards, metabolites were divided into groups belonging to direct or indirect pattern versus metabolites which do not differentiate between both mechanisms.

The pattern for direct effects on the thyroid (based on reference substances inhibiting peroxidase activity) was also confirmed by contrast with changes induced L-thyroxine administration to rats to induce hyperthyroidism (see Supplements Table 1). When the thyroid is indirectly affected, hormone levels are not consistently changed which can make diagnosis of endocrine disruption more difficult. Consideration of additional metabolite patterns can be used to substantiate the suspicion of an indirect hypothyroid effect.

As shown in Table 1 of supplements (A) the typical change of direct acting thyroid toxicants is a profound reduction of thyroxine (T4) in plasma of the animals. This is a classical indicator metabolite for the direct thyroid inhibition MoAs (van Ravenzwaay B. et al., 2012). However, the pattern for the thyroid indirect toxicity shows no consistently decreased T4 (see Table 1 supplements (B)) which is in line with reports by other investigators assessing indirect (liver enzyme induced) thyroid effects in rats (Capen and Martin, 1989).

3.1. Common metabolites altered in males and females in the direct thyroid toxicity pattern

Two metabolites were exclusively and significantly changed in the direct thyroid toxicity pattern in both sexes, the key metabolite T4 was decreased and tricosanoic acid increased.

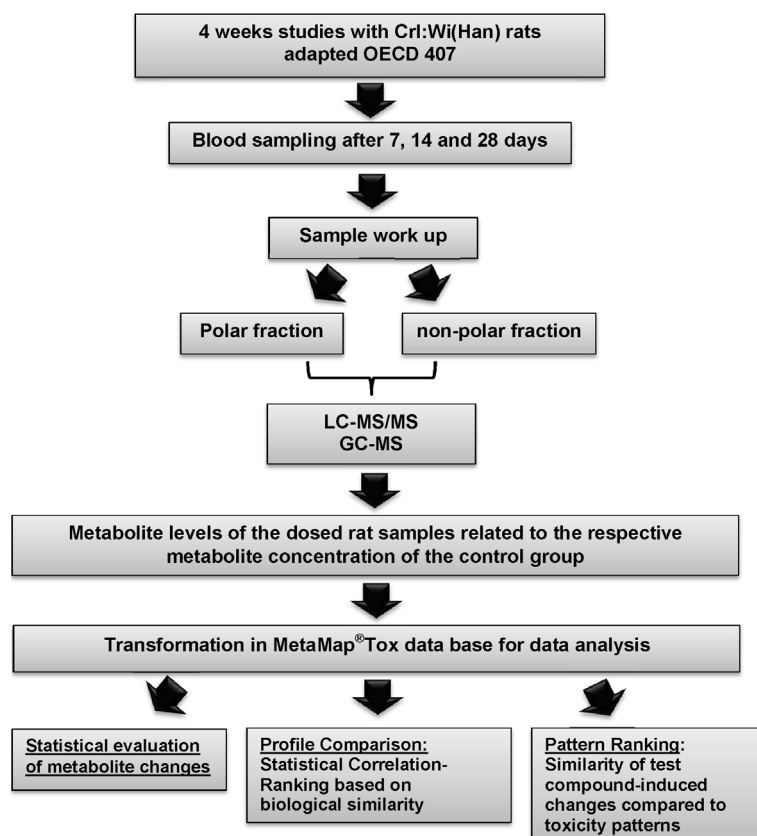


Fig. 2. Flow chart of the methodological process.

PTU, ETU and methimazole inhibit the enzyme thyroperoxidase, which normally acts in thyroid hormone synthesis, facilitating the addition of iodine to tyrosine residues on the hormone precursor thyroglobulin. This is one of the essential steps in the formation of thyroxine (T₄). Significant increase of thyroid-free tyrosine in animals treated with PTU has been due to the decrease of endogenous T₄ production and subsequent stimulation of endogenous TSH release as previously described (Hodge et al., 1969).

It is well known that thyroid hormones are regulators of energy expenditure through activation of β -oxidation of fatty acids (FA) in mammals (Cioffi et al., 2010; Sinha et al., 2012). Odd chain FA accumulation may indicate an activation of ω -oxidation as alternative pathway when β -oxidation is defective, constituting a possible explanation for the up-regulation of tricosanoic acid in the direct thyroid toxicity pattern in mammals including humans (Duran et al., 1984; Sandhir et al., 2000).

Table 2

Changes in body weight and food consumption.^a

Direct Effect	Compound	Direction	Methimazole 100 mg/kg			Ethylenethiourea 300 ppm			6-Propyl-2-thiouracil 100 mg/kg		
			day 7	day 14	day 28	day 7	day 14	day 28	day 7	day 14	day 28
Males	Body Weight	down	0,96	0,90	0,76	0,96	0,94	0,93	0,99	0,98	0,92
	Food Consumption	down	0,77	0,81	0,62	0,90	0,88	0,84	0,96	0,88	0,79
Females	Body Weight	down	0,97	0,96	0,81	1,04	1,01	1,01	0,98	0,97	0,93
	Food Consumption	down	0,68	0,84	0,62	1,04	0,94	0,92	0,99	1,02	0,89

Indirect Effect	Compound	Direction	Boscalid 15000 ppm			Metazachlor 20000 ppm			Aroclor 1254 25 mg/kg			Fipronil 300 ppm			Pendimethalin 10000 ppm		
			day 7	day 14	day 28	day 7	day 14	day 28	day 7	day 14	day 28	day 7	day 14	day 28	day 7	day 14	day 28
Males	Body Weight	down	1,02	1,01	1,00	0,87	0,87	0,90	1,00	0,99	0,99	0,89	0,88	0,94	0,87	0,88	0,82
	Food Consumption	down	1,04	1,04	0,96	0,46	0,79	0,85	0,80	0,75	0,78	0,59	0,93	1,03	0,67	0,85	0,80
Females	Body Weight		1,11	1,09	1,07	0,82	0,83	0,82	1,01	1,05	0,99	0,89	0,93	0,96	0,83	0,85	0,84
	Food Consumption		0,99	1,07	1,07	0,66	0,82	0,87	0,95	0,87	0,86	0,62	1,15	1,04	0,62	0,75	0,78

^a Relative changes in body weight and food consumption of male and female CrI:Wi(Han) rats (N=5 per group and sex) dosed for 4 weeks compared to the corresponding controls. Data were collected on study days 7, 14 and 28, data in red boxes are statistically significantly increased and those in yellow boxes statistically significantly decreased compared to controls (N=10 per group and sex).

3.2. Direct thyroid toxicity pattern in male rats

As shown in Table 3, in male animals, a whole range of metabolites were altered of which 70% are complex lipids, fatty acids and related and 18% are aminoacids and related.

3.2.1. Triglycerides (TAGs), fatty acids (FA) and related

It is known that thyroid function significantly affects lipoprotein metabolism. In humans, decreasing clearance of TAG-rich lipoproteins is found. Therefore, overt hypothyroid patients present elevated TAG levels associated with increased levels of VLDL and occasionally fasting chylomicronemia. In addition, some studies have shown that subclinical hypothyroidism dyslipidemia may also be accompanied by increased TAGs (Abbas et al., 2008; Cohen, 1962; Rizos et al., 2011). However, it has been reported that thyroid hormones could also stimulate lipolysis (Jaworski et al., 2007; Langin, 2006). In rats, treatment with PTU sufficient to lower plasma T3 levels to 60% of normal, and to induce a mild hypothyroidism, also reduces the plasma concentration of VLDL TAGs (Wilcox et al., 1982) as shown in our results (see Table 3). Such apparently contradictory observations suggest that the action of thyroid hormones on lipid metabolism is complex and may, in some aspects, differ from species to species (Dory and Roheim, 1981). In rats, thyroid hormone has been reported to regulate lipoprotein lipase (LPL) in an opposite manner to humans not only in muscle but also in adipose tissue (Coria et al., 2012; Ong et al., 1994).

Fatty acids were particularly changed in males; linolenic acid, isopalmitic acid and 17-methyloctadecanoic acid were down-regulated while lignoceric acid and tricosanoic acid were up-regulated.

α -Linolenic acid 18:3 (*n*-3) is a member of the group of essential fatty acids and is found in many common vegetable oils. Raederstorff et al. (1991) suggested that in hypothyroid rats there is a higher conversion of linolenic into eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Our results showed also DHA up-regulation in male rats treated with methimazole (data not shown) and females (see Table 4) treated with PTU and methimazole.

There is also evidence that mitochondria prepared from thyroidectomized (hypothyroid) rats metabolized palmitic acid at a rate lower than that seen with mitochondria from euthyroid controls in either the fed or fasted state (Pointer et al., 1993). A similar effect could be expected with other FAs like isopalmitic acid and 17-methyloctadecanoic acid.

Aldamiz-Echevarria and col. suggested that plasma concentration of lignoceric acid depends on its β -oxidation both at mitochondrial and peroxisomal levels (Aldamiz-Echevarria et al., 2009) and β -oxidation is stimulated by thyroid hormones in mammals (Sinha et al., 2012).

3.2.2. Sphingolipids and sphingomyelins

Hypothyroidism is characterized by essential disorders of the phospholipid metabolism which occur already at the initial stages of the disease, advances with progression of its severity and is manifested in an increase of phosphatidylserine, lysophosphatidylcholine, sphingomyelin and phosphatidylethanolamine (Zelinskaia, 1989). These results are in line with those obtained in the study (see Table 3). Changes in the phospholipid components can be due to thyroid hormone effects on desaturases, phospholipases, acyltransferases or fatty acid catabolism in humans and rodents (Babenko and Natarova, 1999; Faas and Carter, 1982; Gredilla et al., 2001).

3.2.3. Amino acids

Alterations in various amino acid levels were also observed in this pattern for males: tryptophan, arginine and tyrosine were up-regulated while glutamate was down-regulated. It is worth

mentioning, that these amino acids are oppositely regulated in the pattern for indirect thyroid toxicity (see Table 7). Thyroid hormones are able to stimulate gene expression and transcriptional synthesis of proteins in mammals (Muller and Seitz, 1984) including gluconeogenesis, lipogenesis, insulin signaling, adenylate cyclase signaling, cell proliferation, and apoptosis (Feng et al., 2000). Thus, hypothyroidism can provoke an increase of proteolysis and an inhibition of the utilization of amino acids for protein synthesis; as a result, there are higher amino acid levels in plasma (Remesar et al., 1981; Hayase et al., 1993; Rochon et al., 2000).

Transamination of α -ketoglutarate (an intermediate in the citric acid cycle) results in glutamate. The α -ketoacid product contributes as a substrate for further metabolism processes such as glycolysis and gluconeogenesis. There is some evidence that thyroid hormones promote the development of enzymes of ketone-body metabolism at least in rats (Diez-Guerra et al., 1980), and this, together with the above mentioned effect regarding the decreased energy metabolism in hypothyroidism, could justify the observed down-regulation of glutamate in this pattern. Furthermore, decrease of muscular effluxes of glutamine from glutamate in hypothyroid rats has been previously described (Parry-Billings et al., 1990). This effect can be explained with a decrease of the glutamate/aspartate transporter (GLAST) and glutamate transporter-1 (GLT-1) (Zamoner et al., 2008). As oxoproline is a derivative of the free amino group of glutamic acid or glutamine, its down-regulation correlates with the effect of thyroid hormones on glutamate.

Thyroid hormones also reduce urea synthesis and excretion. Treatment with PTU increases carbamylphosphate synthetase and ornithine carbamyl transferase, while the arginine synthetase system and arginase are unchanged. According to our experiments, urea, citrulline and ornithine were up-regulated in male and female rats; these data match well with the literature (Hayase et al., 1993; Grillo and Fossa, 1966).

Hypothyroidism has negative influence on kidney function in mammals, indirectly by affecting the cardiovascular system and the renal blood flow and directly by affecting glomerular filtration, tubular functions and the structure of the kidney. Thus this condition is associated with consistent reversible elevation in the serum creatinine and urea levels (Kreisman and Hennessey, 1999; Salama et al., 2012). Salama et al. (2012) showed that PTU induces variable pathological changes in glomeruli and some parts of the urinary tubules in the kidney of hypothyroid rats and moreover, several case reports have described myalgia and elevations of serum creatine kinase (an enzyme that catalyses the conversion of creatine to phosphocreatine) in patients with Graves' disease undergoing antithyroid drugs therapy (Chieh-Hua and Chang-Hsun, 2007). Serum creatine kinase levels show an inverse relation with serum T3 and T4 levels in humans (Prakash et al., 2007) and L-arginine:glycine amidinotransferase (AGAT – an enzyme that catalyses the transfer of an amidino group from L-arginine to glycine) activity in the kidney is also potently inhibited by ornithine (Wyss and Kaddurah-Daouk, 2000). The direct effects of treatment with PTU and methimazole on urea cycle are illustrated in Fig. 3.

3.2.4. Other metabolites

As previously published, campesterol is a biomarker related to food consumption in rats. Its plasma concentrations are decreased in rats fed a high caloric diet correlating with decreased food consumption in these animals (Mellert et al., 2011). Thyroid dysfunction can have clinically significant consequences on food consumption and body weight. Hypothyroidism classically causes reduced basal energy expenditure with weight gain (Amin et al., 2011). However in our experiments, food consumption was significantly reduced while plasma campesterol concentrations were

Table 3
Metabolite pattern for direct thyroid toxicity for male rats.

Compound	Direction	Class	Subclass
Glutamate	Down	Amino acids	Amino acids, acidic
Tryptophan	Up	Amino acids	Amino acids, aromatic
Tyrosine	Up	Amino acids	Amino acids, aromatic
Arginine	Up	Amino acids	Amino acids, basic
5-Oxoproline (additional: folic acid, glutamate, glutamine)	Down	Amino acids related	Amino acid metabolites
Creatinine (additional: creatine, phosphocreatine)	Down	Amino acids related	Creatine metabolism
DAG (C18:1,C18:2)	Down	Complex lipids, fatty acids and related	Diacylglycerols
17-Methyloctadecanoic acid	Down	Complex lipids, fatty acids and related	Fatty acids, branched
Linolenic acid (C18:cis[9,12,15]3)	Down	Complex lipids, fatty acids and related	Fatty acids, poly-unsaturated
Dihomo- γ -linolenic acid (C20:cis[8,11,14]3)	Up	Complex lipids, fatty acids and related	Fatty acids, poly-unsaturated
Lignoceric acid (C24:0)	Up	Complex lipids, fatty acids and related	Fatty acids, saturated
Tricosanoic acid (C23:0)	Up	Complex lipids, fatty acids and related	Fatty acids, saturated
Isopalmitic acid (C16:0)	Down	Complex lipids, fatty acids and related	Fatty acids, saturated
4-Hydroxysphinganine (t18:0, phytosphingosine), total	Up	Complex lipids, fatty acids and related	Sphingolipids
3-O-methylsphingosine (d18:1) (additional: sphingolipids, erythro-sphingosine (d18:1), threo-sphingosine (d18:1))	Up	Complex lipids, fatty acids and related	Sphingolipids
Threo-sphingosine (d18:1) (additional: sphingolipids)	Up	Complex lipids, fatty acids and related	Sphingolipids
5-O-methylsphingosine (d18:1)	Up	Complex lipids, fatty acids and related	Sphingolipids
Erythro-sphingosine (d18:1) (additional: sphingolipids) (additional: sphingolipids)	Up	Complex lipids, fatty acids and related	Sphingolipids
Sphingomyelin (d18:2,C16:0)	Up	Complex lipids, fatty acids and related	Sphingomyelins
Sphingomyelin (d18:1,C16:0)	Up	Complex lipids, fatty acids and related	Sphingomyelins
Sphingomyelin (d18:1,C16:0)	Up	Complex lipids, fatty acids and related	Sphingomyelins
Cholesterol, total	Up	Complex lipids, fatty acids and related	Steroid
TAG (C18:2,C18:2)	Down	Complex lipids, fatty acids and related	Triacylglycerols
TAG (C16:0,C18:2)	Down	Complex lipids, fatty acids and related	Triacylglycerols
TAG (C18:1,C18:2)	Down	Complex lipids, fatty acids and related	Triacylglycerols
TAG (C16:0,C16:1)	Down	Complex lipids, fatty acids and related	Triacylglycerols
TAG (C18:2,C18:3)	Down	Complex lipids, fatty acids and related	Triacylglycerols
TAG (C18:2,C18:2)	Down	Complex lipids, fatty acids and related	Triacylglycerols
TAG (C18:1,C18:2,C18:3) (additional: TAG (C16:0,C18:1,C20:5), TAG (C16:0,C18:2,C20:4))	Down	Complex lipids, fatty acids and related	Triacylglycerols
Citrate	Down	Energy metabolism and related	Citrate cycle
Thyroxine (t4)	Down	Hormones, signal substances and related	Signal substances
Campesterol	Up	Miscellaneous	Diet related
Alpha-tocopherol	Up	Vitamins, cofactors and related	Tocopherols and related

up-regulated in male and female rats in comparison to control animals. A competition of absorption in the intestinal lumen between the phytosterols and cholesterol has been previously described (Mellert et al., 2011; Ostlund, Jr., 2007). Since cholesterol plasma concentrations were up-regulated, ABC transporters (ABCG5 and ABCG8) which regulate intestinal sterol absorption, could pump

cholesterol selectively back from the enterocyte into the intestinal lumen thereby regulating their absorption rates in humans (Sudhop et al., 2002).

As for alpha-tocopherol, it is well known that the concentration of this vitamin in the thyroid of normal rats is almost as high as in liver, and higher than in plasma. It decreases during iodine

Table 4
Metabolite pattern for direct thyroid toxicity for female rats.

Compound	Direction	Class	Subclass
Asparagine	Up	Amino acids	Amino acids, basic
Glycine	Up	Amino acids	Amino acids, neutral
Ketoleucine	Down	Amino acids related	Amino acid metabolites
Docosahexaenoic acid (C22:cis[4,7,10,13,16,19]6)	Up	Complex lipids, fatty acids and related	Fatty acids, poly-unsaturated
Tricosanoic acid (C23:0)	Up	Complex lipids, fatty acids and related	Fatty acids, saturated
Behenic acid (C22:0)	Up	Complex lipids, fatty acids and related	Fatty acids, saturated
Lysophosphatidylcholine (C18:2)	Up	Complex lipids, fatty acids and related	Lysophosphatidylcholines
Phosphatidylcholine (C16:1,C18:2)	Up	Complex lipids, fatty acids and related	Phosphatidylcholines
Phosphatidylcholine (C16:0,C22:6) (additional: phosphatidylcholine (C18:2,C20:4))	Up	Complex lipids, fatty acids and related	Phosphatidylcholines
Threo-sphingosine (d18:1) (additional: sphingolipids)	Up	Complex lipids, fatty acids and related	Sphingolipids
5-O-methylsphingosine (d18:1) (additional: sphingolipids, erythro-sphingosine (d18:1), threo-sphingosine (d18:1))	Up	Complex lipids, fatty acids and related	Sphingolipids
Erythro-sphingosine (d18:1) (additional: sphingolipids)	Up	Complex lipids, fatty acids and related	Sphingolipids
Sphingomyelin (d18:1,C16:0)	Up	Complex lipids, fatty acids and related	Sphingomyelins
Sphingomyelin (d18:1,C16:0)	Up	Complex lipids, fatty acids and related	Sphingomyelins
Sphingomyelin (d18:1,C24:0)	Up	Complex lipids, fatty acids and related	Sphingomyelins
TAG (C16:0,C18:1,C18:3) (additional: TAG (C16:0,C18:2,C18:2), TAG (C16:1,C18:1,C18:2))	Down	Complex lipids, fatty acids and related	Triacylglycerols
Citrate	Down	Energy metabolism and related	Citrate cycle
Pyruvate (additional: phosphoenolpyruvate (PEP))	Down	Energy metabolism and related	Glycolysis/gluconeogenesis
thyroxine (T4)	Down	Hormones, signal substances and related	Signal substances and related
18-Hydroxy-11-deoxycorticosterone (additional: 11-deoxycortisol)	Down	Hormones, signal substances and related	Steroids and related

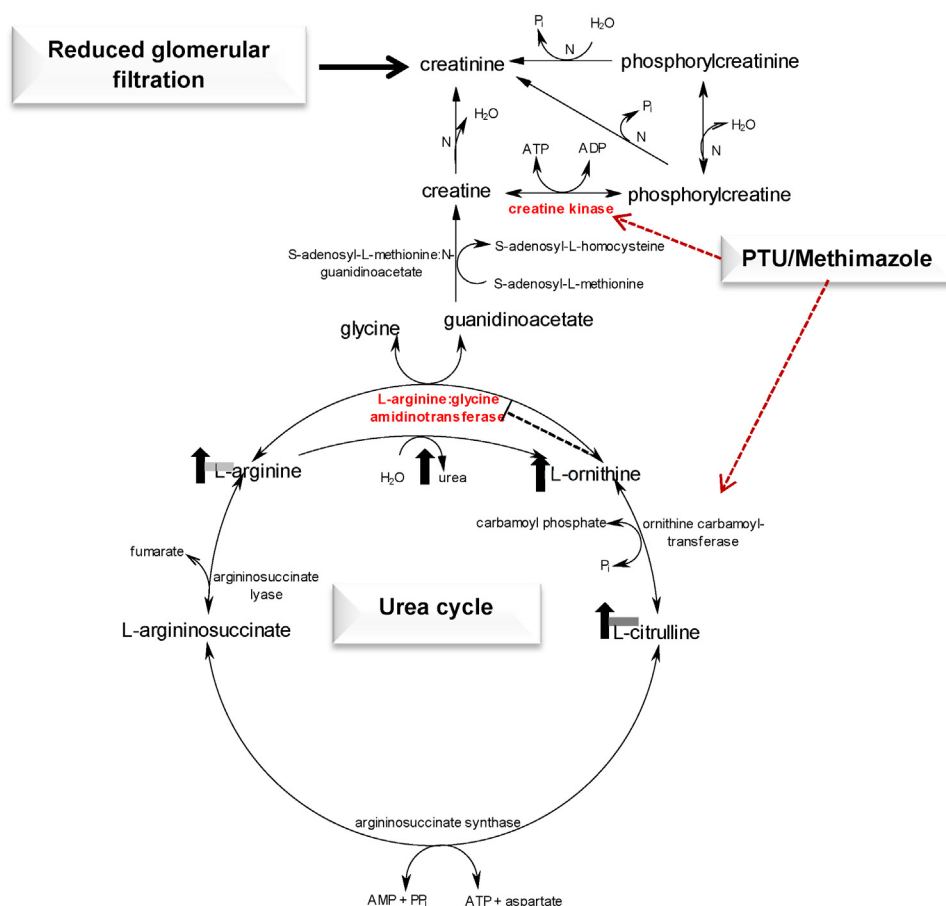


Fig. 3. Schematic representation of the effects of thionamides on reactions and enzymes involved in creatine and creatinine metabolism. Dark black arrows: direction of metabolic regulation in the direct thyroid toxicity pattern in male rats, red dashed arrows: enzyme activity stimulation and black dashed T-bars: enzyme activity inhibition. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Adapted from Wyss and Kaddurah-Daouk (2000).

deficiency in the liver while it increases in thyroid and plasma. Total lipid content is raised in serum during hypothyroidism and a high correlation between serum total lipids and tocopherol levels exists in rats and humans (Mutaku et al., 1998; Duntas and Orgiazzi, 2003).

3.3. Direct thyroid toxicity pattern in female rats

Metabolite changes in female rats are shown in Table 4, 62% belong to the group complex lipids and the remaining are aminoacids, hormones and energy metabolites.

Many of the altered metabolites in females were also changed in the same direction but not significantly in males (data not shown), pathway explanations given above apply to many of the metabolites as amino acids and lipids in the pattern of females.

Additionally, pyruvate, a key energy metabolite, was down-regulated in the direct thyroid toxicity pattern. As mentioned above, this MoA is characterized by a generally decreased energy metabolism (decreased β -oxidation, tricarboxylic acid cycle and triglyceride synthesis) due to the severely reduced thyroid hormone levels. Additionally, PDK4 (pyruvate dehydrogenase kinase 4) regulates pyruvate oxidation through the phosphorylation and inhibition of the pyruvate dehydrogenase complex (PDC). PDC catalyze the conversion of pyruvate to acetyl-CoA and is an important control point in glucose and pyruvate metabolism. It is worth noting that PDK4 gene expression is stimulated by thyroid hormones in humans (Attia et al., 2010).

Ketoleucine, a metabolite derived from the transamination of leucine, plays a unique role in branched-chain ketoacid catabolism of the liver in rats (Baranyai and Blum, 1989). Studies show that during hypothyroidism whole body leucine flux and protein synthesis are decreased in humans (Rochon et al., 2000), and this is in line with the down-regulation of ketoleucine in our direct thyroid toxicity pattern for females.

Finally, the down-regulation of 18-hydroxy-11-deoxycorticosterone could be due to the decrease of metabolic clearance and production of cortisol in hypothyroidism. Neri et al. indicated that thyrotropin-releasing hormone (TRH) markedly inhibits glucocorticoid secretion of rat adrenocortical cells, which selectively impairs the late steps of corticosterone synthesis (i.e., 11- and 18-hydroxylation) (Neri et al., 1993). Lo et al. (1998) suggested that the reduction of plasma corticosterone in PTU-treated rats may be due to the toxic effect of PTU on the adrenal gland.

3.4. Indirect thyroid toxicity pattern in male rats

It is important to recognize whether a novel compound causes hepatic microsomal enzyme induction, because such compounds can alter their own metabolism and can modify the rate of thyroid hormone excretion (Davies, 1993). An increased clearance of T4 for example, results in lower circulating T4 values and a positive feedback at the pituitary. This allows an increased release of TSH and the turnover of both TSH and T4 could be also significantly higher.

Table 5
Metabolite pattern for indirect thyroid toxicity for male rats.

Compound	Direction	Class	Subclass
Glycine	Up	Amino acids	Amino acids, neutral
Mannose	Up	Carbohydrates and related	Monosaccharides
Nervonic acid (C24:cis[15]1)	Up	Complex lipids, fatty acids and related	Fatty acids, mono-unsaturated
Dihomo-gamma-linolenic acid (C20:cis[8,11,14]3)	Up	Complex lipids, fatty acids and related	Fatty acids, poly-unsaturated
Stearic acid (C18:0)	Up	Complex lipids, fatty acids and related	Fatty acids, saturated
Lignoceric acid (C24:0)	Up	Complex lipids, fatty acids and related	Fatty acids, saturated
Behenic acid (C22:0)	Up	Complex lipids, fatty acids and related	Fatty acids, saturated
Galactose, lipid fraction	Up	Complex lipids, fatty acids and related	Glycolipids
Lysophosphatidylcholine (C18:0)	Up	Complex lipids, fatty acids and related	Lysophosphatidylcholines
Phosphatidylcholine (C18:0,C18:2)	Up	Complex lipids, fatty acids and related	Phosphatidylcholines
Phosphatidylcholine (C18:0,C18:1)	Up	Complex lipids, fatty acids and related	Phosphatidylcholines
Glycerol phosphate, lipid fraction	Up	Complex lipids, fatty acids and related	Phospholipid metabolites
Myo-Inositol-2-phosphate, lipid fraction (myo-inositolphospholipids)	Up	Complex lipids, fatty acids and related	Phospholipid metabolites
3-O-Methylsphingosine (d18:1) (additional: sphingolipids, erythro-sphingosine (d18:1), threo-sphingosine (d18:1))	Up	Complex lipids, fatty acids and related	Sphingolipids
Threo-sphingosine (d18:1) (additional: sphingolipids)	Up	Complex lipids, fatty acids and related	sphingolipids
5-O-Methylsphingosine (d18:1) (additional: sphingolipids, erythro-sphingosine (d18:1), threo-sphingosine (d18:1))	Up	Complex lipids, fatty acids and related	Sphingolipids
Erythro-sphingosine (d18:1) (additional: sphingolipids)	Up	Complex lipids, fatty acids and related	Sphingolipids
Sphingomyelin (d18:1,C16:0)	Up	Complex lipids, fatty acids and related	Sphingomyelins
Sphingomyelin (d18:1,C16:0)	Up	Complex lipids, fatty acids and related	Sphingomyelins
Cholesterol, total	Up	Complex lipids, fatty acids and related	Steroids and related
Citrate	Down	Energy metabolism and related	Citrate cycle
Cytosine (additional: 2'-deoxycytidine)	Down	Nucleobases and related	Pyrimidine metabolism
Threonic acid	Up	Vitamins, cofactors and related	Ascorbic acid and related

As shown in Table 5, in male animals, the majority (78.3%) of the altered metabolites belongs to the complex lipids, fatty acids and related group, such as phospholipid metabolites (myo-Inositol-2-phosphate and glycerol phosphate), fatty acids (nervonic acid, stearic acid and arachidonic acid), phosphatidylcholines (C18:0, C18:2 and C18:0, C18:1), sphingolipids

(4-hydroxysphinganine) and ceramides (see Table 5). In contrast to the direct pattern, thyroxine levels were not consistently decreased but liver lipid metabolism was consistently enhanced (increased fatty acid levels). Therewith, this profile fits well with typical metabolite changes in profiles correlated to liver toxicity.

Table 6
Metabolite pattern for indirect thyroid toxicity for female rats.

Compound	Direction	Class	Subclass
Threonine	Up	Amino acids	Amino acids, neutral
Cysteine	Down	Amino acids	Amino acids, s-containing
Ceramide (d18:1,C24:0)	Up	Complex lipids, fatty acids and related	Ceramides
DAG (C18:1,C18:2)	Up	Complex lipids, fatty acids and related	Diacylglycerols
Nervonic acid (C24:cis[15]1)	Up	Complex lipids, fatty acids and related	Fatty acids, mono-unsaturated
linoleic acid (C18:cis[9,12]2)	Up	Complex lipids, fatty acids and related	Fatty acids, poly-unsaturated
Arachidonic acid (C20:cis[5,8,11,14]4)	Up	Complex lipids, fatty acids and related	Fatty acids, poly-unsaturated
Docosahexaenoic acid (C22:cis[4,7,10,13,16,19]6)	Up	Complex lipids, fatty acids and related	Fatty acids, poly-unsaturated
Gamma-linolenic acid (C18:cis[6,9,12]3)	Up	Complex lipids, fatty acids and related	Fatty acids, poly-unsaturated
Dihomo-gamma-linolenic acid (C20:cis[8,11,14]3)	Up	Complex lipids, fatty acids and related	Fatty acids, poly-unsaturated
Palmitic acid (C16:0)	Up	Complex lipids, fatty acids and related	Fatty acids, saturated
Stearic acid (C18:0)	Up	Complex lipids, fatty acids and related	Fatty acids, saturated
Heptadecanoic acid (C17:0)	Up	Complex lipids, fatty acids and related	Fatty acids, saturated
Eicosanoic acid (C20:0)	Up	Complex lipids, fatty acids and related	Fatty acids, saturated
Behenic acid (C22:0)	Up	Complex lipids, fatty acids and related	Fatty acids, saturated
Dodecanol	Up	Complex lipids, fatty acids and related	Fatty alcohols
Glycerol-3-phosphate, polar fraction	Up	Complex lipids, fatty acids and related	Lipid precursors
Lysophosphatidylcholine (C17:0)	Up	Complex lipids, fatty acids and related	Lysophosphatidylcholines
Phosphatidylcholine (C18:0,C18:2)	Up	Complex lipids, fatty acids and related	Phosphatidylcholines
Phosphatidylcholine (C18:0,C18:1)	Up	Complex lipids, fatty acids and related	Phosphatidylcholines
Glycerol phosphate, lipid fraction	Up	Complex lipids, fatty acids and related	Phospholipid metabolites
Myo-Inositol-2-phosphate, lipid fraction (myo-inositolphospholipids)	Up	Complex lipids, fatty acids and related	Phospholipid metabolites
4-Hydroxysphinganine (t18:0, Phytosphingosine), total	Up	Complex lipids, fatty acids and related	Sphingolipids
3-O-methylsphingosine (d18:1) (additional: sphingolipids, erythro-sphingosine (d18:1), threo-sphingosine (d18:1))	Up	Complex lipids, fatty acids and related	Sphingolipids
threo-sphingosine (d18:1) (additional: sphingolipids)	Up	Complex lipids, fatty acids and related	Sphingolipids
5-O-methylsphingosine (d18:1) (additional: sphingolipids, erythro-sphingosine (d18:1), threo-sphingosine (d18:1))	Up	Complex lipids, fatty acids and related	Sphingolipids
Erythro-sphingosine (d18:1)(additional: sphingolipids)	Up	Complex lipids, fatty acids and related	Sphingolipids
Sphingomyelin (d18:1,C16:0)	Up	Complex lipids, fatty acids and related	Sphingomyelins
Sphingomyelin (d18:2,C18:0)	Up	Complex lipids, fatty acids and related	Sphingomyelins
Sphingomyelin (d18:1,C16:0)	Up	Complex lipids, fatty acids and related	Sphingomyelins
Sphingomyelin (d18:1,C24:0)	Up	Complex lipids, fatty acids and related	Sphingomyelins
Cholesterol, total	Up	Complex lipids, fatty acids and related	Steroids and related
Threonic acid	Up	Vitamins, cofactors and related	Ascorbic acid and related

Table 7

Metabolites regulated oppositely in patterns for indirect and direct thyroid toxicity for male and female rats.

Compound	Class	Subclass	Direct effect ^a		Indirect effect ^a	
			Males	Females	Males	Females
DAG (C18:1,C18:2)	Complex lipids, fatty acids and related	Diacylglycerols	Down	Upward trend	Downward trend	Up
17-Methyloctadecanoic acid	Complex lipids, fatty acids and related	Fatty acids, saturated	Down	Downward trend	Upward trend	Up
glutamate	Amino acids	Amino acids, acidic	Down	Downward trend	Upward trend	Upward trend
Tryptophan	Amino acids	Amino acids, aromatic	Up	Downward trend	Downward trend	Downward trend
Tyrosine	Amino acids	Amino acids, aromatic	Up	Upward trend	Downward trend	Downward trend
Arginine	Amino acids	Amino acids, basic	Up	Downward trend	Downward trend	Downward trend
TAG (C18:2,C18:2)	Complex lipids, fatty acids and related	Triacylglycerols	Down	Downward trend	Upward trend	Upward trend
TAG (C16:0,C18:2)	Complex lipids, fatty acids and related	Triacylglycerols	Down	Downward trend	Upward trend	Upward trend
TAG (C18:1,C18:2)	Complex lipids, fatty acids and related	Triacylglycerols	Down	Downward trend	Upward trend	Upward trend
TAG (C16:0,C18:1,C18:3)	Complex lipids, fatty acids and related	Triacylglycerols	Downward trend	Down	Upward trend	Upward trend

^a Upward/downward trend means increased/decreased metabolite levels in dosed animals compared to controls, but not significant difference for each reference compound or each blood sampling days.

While the thyroid gland is a major target organ of toxicity, effects have also been observed in the liver. As previously published in our group (van Ravenzwaay B. et al., 2012), we demonstrated that effects on the liver (enzyme induction and liver toxicity) can be linked to metabolome patterns, being representative for indirect thyroid toxicity: Liver associated patterns showed some similarities suggesting that the initial effect of some of the liver and thyroid toxicities are based on hepatic enzyme induction. For example metabolites as glycerol, myo-inositol-2-phosphate, palmitic acid, arachidonic acid, cholesterol, lignoceric acid and behenic acid are similarly changed in the same manner in liver and thyroid toxicity patterns in rats.

3.5. Indirect thyroid toxicity pattern in female rats

As shown in Table 6, 90.9% of the altered metabolites are lipids and related. As aforementioned these metabolite changes are strongly related with liver enzyme induction and liver toxicity patterns.

Regarding phosphatidylcholines, metabolites that were specifically changed in the indirect thyroid toxicity pattern, there is some evidence that long chain FA stimulate the hepatic biosynthesis in rats (Pelech et al., 1983). It has been suggested that alterations in the rate of phosphatidylcholine synthesis correlate with changes in the activity of phosphocholine cytidyltransferase (CTP) that could be strongly induced by FA such as arachidonic acid, oleic acid and palmitic acid. Considering that these FA were also up-regulated in the indirect thyroid toxicity pattern (especially in females, see Table 6) it could be one explanation for the regulation of this metabolite group. However, it is worth noting that other authors described an inhibition of the CTP-activity by sphingosines in rats (Sohal and Cornell, 1990).

3.6. Metabolites commonly altered in direct and indirect patterns in both sexes

Metabolites such as cholesterol, dihomogamma-linolenic acid, sphingosines (3-O-methylsphingosine, threo-sphingosine, 5-O-methylsphingosine and erythro-sphingosine), sphingomyelins (d18:2,C16:0 and d18:1,C16:0), 4-hydroxysphinganine, behenic acid and citrate were changed significantly in the same manner in males and females in both patterns (direct and indirect thyroid toxicity).

The predominant regulation of metabolites such as citrate, fatty acids and cholesterol in the thyroid toxicity patterns could be explained as being a result of a general decreased energy metabolism (decreased β -oxidation, tricarboxylic acid cycle and triglyceride synthesis) due to the alterations in thyroid hormone homeostasis which is true for all mammals. Thus, thyroid hypofunction is characterized by impaired glucose absorption from the gastrointestinal tract and delayed peripheral gluconeogenesis, decreased or normal hepatic glucose output and decreased peripheral tissue glucose disposal (Duntas et al., 2011). Hypothyroidism in rats decreased the maximum activities of both intestinal citrate synthase and oxoglutarate dehydrogenase, suggesting a decreased intestinal capacity for the tricarboxylic acid cycle under these conditions (Ardawi and Jalalah, 1991; Ardawi, 1991). There is also evidence that succinoxidase activity in some mammalian tissues is depressed by thyroidectomy and increased by administration of thyroid hormones (Cohen, 1962).

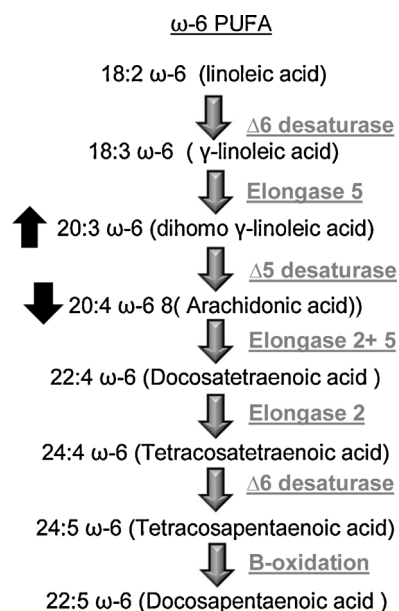


Fig. 4. Linoleic acid metabolism. Schematic representation of the metabolism of ω -6 PUFAs. Dark black arrows = direction of metabolic regulation in male and female rats treated with PTU.

Adapted from Igarashi et al. (2007).

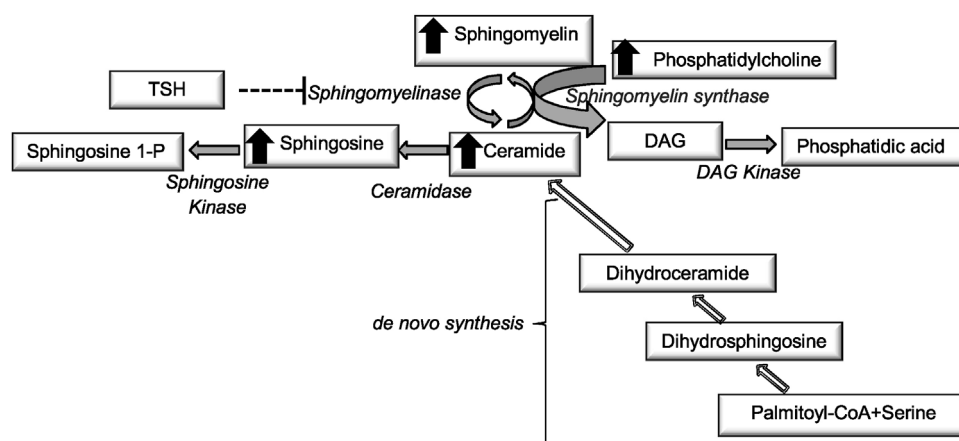


Fig. 5. Ceramide pathway. Ceramide can be formed *de novo* or from hydrolysis of sphingomyelin or complex glycolipids. Phosphatidylcholine is used to convert ceramide to sphingomyelin in the sphingomyelin cycle. Dark black arrows: direction of metabolic regulation in the indirect thyroid toxicity pattern, black dashed T-bars: enzyme activity inhibition.

Adapted from Hannun and Wang (Hannun and Obeid, 2002, 2008; Wang and Bieberich, 2010; Direito et al., 2009).

There is some evidence of the up-regulation of dihomogamma-linolenic acid in hypothyroid rats (Raederstorff et al., 1991). Additionally the $\Delta 6$ and $\Delta 5$ desaturases involved in the conversion of 18:2 n -6 (linoleic acid) into 20:4 n -6 (arachidonic acid) were found to be decreased in hypothyroid rats (Hoch, 1981) and mice (Guerrero et al., 1999) compared to control group when n -6 fatty acids were used as substrates. There is also evidence that hypothyroidism changes fatty acid levels in plasma, erythrocytes and polymorphonuclear leukocytes, suggesting a disturbance in the $\Delta 6$ desaturase activity (van Doormaal et al., 1986). This effect is also described in polymorphonuclear leukocytes and the heart of rats (Coria et al., 2012; Hamplova et al., 2003). Down-regulation of arachidonic acid together with an up-regulation of dihomogamma-linolenic acid was observed (data not shown) for the male and female rats treated with PTU. Such disturbances in the $\Delta 5$ and $\Delta 6$ desaturases activities (see Fig. 4) could be considered as an explanation for the up-regulation of the dihomogamma-linolenic acid for a general thyroid toxicity pattern.

It is well known that thyroid hormones induce the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, which is the first step in cholesterol biosynthesis in mammals (Duntas, 2002; Duntas et al., 2011; Rizos et al., 2011). However, there is evidence that in spite of the reduced activity of HMG-CoA reductase, total cholesterol levels are increased in hypothyroidism (Pearce et al., 2008a,b; Rizos et al., 2011). This is due to the decreased LDL-receptors' activity, resulting in decreased catabolism of LDL (low-density lipoprotein) a cholesterol-rich lipoprotein and IDL (intermediate-density lipoprotein). Moreover, a decreased activity of LPL (lipoprotein lipase), a water soluble enzyme that hydrolyzes triglycerides from lipoproteins and also promotes the cellular uptake of chylomicron remnants, LDL and IDL, is found in overt hypothyroidism (Rizos et al., 2011).

In the same manner, there is evidence that sphingomyelin hydrolysis is down-regulated by TSH through a direct inhibition of sphingomyelinase in humans (Schneider et al., 2000). This leads to an increase of the sphingomyelin and should result in a decrease of the ceramide concentrations. However, ceramide was up-regulated particularly by ETU and methimazole in the direct patterns and by all test compounds in the indirect patterns (data not shown). Ceramide accumulation is predominantly induced by an activation of the *de novo* pathway or an inhibition of ceramide utilization, see Fig. 5.

In the sphingolipid pathway ceramide can be phosphorylated, glycosylated or can receive a phosphocholine headgroup from phosphatidylcholine in the biosynthesis of sphingomyelin through

the action of sphingomyelin synthase in rats and humans, see Fig. 5 (Claus et al., 2009; Hannun and Obeid, 2002; Direito et al., 2009; Hannun and Obeid, 2008). It can be assumed that the sphingomyelin synthase is also inhibited by high sphingomyelin levels leading to an increase of the phosphatidylcholines.

Recently, Wu et al. applied a LC/MS metabonomic method in urine samples to show that hypothyroidism primarily affected energy, amino acid, sphingolipid and purine metabolism. They found a dramatic decrease of ceramide and dihydrosphingosine in urine (Wu et al., 2013). Taking into account that changes in the rat's sphingolipid metabolism could be detected in urine (Direito et al., 2009) and that under hypothyroidic conditions the glomerular filtration rate is reduced, our results matched well with the findings of Wu et al.

It can be concluded that it is possible to distinguish different MoAs resulting in hypothyroidism in the rat, using metabolic profiling (metabolomics) in blood. Compound induced toxicity patterns for direct and indirect thyroid effects were well defined for males and females and the biochemical explanation of characteristic changes of endogenous metabolites (see Table 7) provide strong coherence with biochemical and toxicological mechanisms through which these compounds act.

Some mentioned mechanisms can be assumed to occur in mammals including humans with hypothyroidism, i.e., metabolite level changes due to a decreased energy metabolism and protein synthesis, alterations of the urea cycle as well as specific increases of cholesterol, tyrosine and tricosanoic acid levels. Because of species-specific differences especially in the lipid metabolism as well as in the enzyme pattern responsible for liver enzyme induction, different regulation of involved metabolites during hypothyroidism can be expected, as mentioned for increased TAG levels in humans, but decreased TAGs values in the direct thyroid toxicity patterns in rats. Also, the described mechanisms leading to decreased glutamate and 18-hydroxy-corticosterone levels were assumed to be specific for rodents with hypothyroidism.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.toxlet.2013.12.010>.

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