

# The immunosenescence and lipotoxicity in thyrotoxicosis mice

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## Article

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## Abstract

COVID-19 is a worldwide outbreak now, and it is found to be age-related. Immunosenescence may be a predisposing and severe factor for COVID-19. Besides, many infectious diseases in clinic are age-related, and elderly patients have longer hospitalization and worse prognosis. Therefore, finding suitable aging models is of great significance for fighting aging related diseases and promoting the prognosis of elderly patients.

In this study, the relationship between thyrotoxicosis and aging was investigated by routine detection and serum metabolomics in mice. The results of routine blood test and flow cytometry showed significant decrease in neutrophils, lymphocytes, CD4+/CD8+ and CD4+IFN- $\gamma$  + lymphocytes in thyrotoxicosis mice. Biochemical examination combined with serum metabolomics analysis showed that serious disorder of lipid metabolism may be one of the causes of immunosenescence, including lower cholesterol levels, lower levels of VD and bile acids, high level of glucocorticoids, triglycerides, free fatty acids, Sphingolipids and decrease of Docosanoids, especially DPA.

This study proves that thyrotoxicosis mice are an accelerated aging model. In present study, the main performance is immunosenescence, which may be due to lipotoxicity, suggesting that the immunosenescence state can be adjusted by improving lipotoxicity, whether anti thyroxine or not. However, there are other manifestations of thyroid toxicity mouse model simulating aging, such as organ aging, which need to continue to be studied by means of system biology to provide more comprehensive evidence.

## Main

Thyroid hormones play a very important role in almost all body tissues by regulates metabolism, growth and development <sup>1</sup>. The homeostasis of serum thyroxine is under precisely monitored and regulated. The interference of homeostasis by external or internal factors will lead to hyperthyroidism or hypothyroidism. One of the characteristics of aging is the significant increase in thyroid disease, and the prevalence of hyperthyroidism has increased by 7 times in people over 65 years old <sup>2</sup>. More than that, hyperthyroidism can mimic many other diseases. Untreated hyperthyroidism can lead to various systemic problems, including heart, bone, muscle, as well as cognitive impairment <sup>3</sup>. All these diseases are related with aging, as well as infectious diseases, such as COVID-19, and biological age is more relevant than actual age <sup>4</sup>. Actually, not only aging increases the incidence rate of hyperthyroidism, but hyperthyroidism also accelerates biological aging. Thyroid hormones increase the energy consumption of most tissues of the whole body, which is related to accelerated kinetics <sup>5</sup>. Accelerating metabolism is bound to bring a series of chain reactions, which will lead to the changes of metabolites, and have a great impact on the internal environmental homeostasis, leading to the increase of aging related factors. Current studies have focused on the thermogenic reaction of thyroid hormones on brown and/or white adipose tissue, and there are few studies on the changes of lipid metabolites in this process <sup>6</sup>. In fact, the impact of metabolites on the body should not be underestimated, and there is still less systematic description about the metabolites in hyperthyroidism, and whether these factors are related to aging.

As well known, immune dysfunction is common feature of aging, age related immune system remodeling or immunosenescence are considered to be the main causes of increased susceptibility to infection, loss of control of persistent infections, poorer responses to vaccination, lower capacity to mediate anti-cancer responses <sup>7</sup>. For example, several aspects of neutrophil response are affected by normal aging, including traditional neutrophil functions, such as phagocytosis and oxidative outbreaks. Because they never divide and have little capacity for self-repair, they are susceptible to dead when activated or damaged, so if there is not enough supplement, it is easy to cause a decline in quantity. These changes reduce the ability to eliminate bacteria and fungi, inhibited the interaction with adaptive immune system and affect the adaptive immune system, leading to decline of immunity <sup>8</sup>. Also, the decrease of CD4+/CD8+ ratio is one of the markers of T cell "immunosenescence". The reverse of CD4+:CD8+ ratio in healthy elderly population is due

to the decrease of CD4+T cell count and the increase of CD8+T cell count, which was associated with poor immunity, increased morbidity and increased mortality<sup>9</sup>. Immunosenescence not only occurs in the elderly, but also is a marker of AIDS immune damage, and is related to the prognosis of AIDS<sup>10</sup>. Additionally, it is of great significance to evaluate the host immune function by combining the number and function of lymphocytes for the diagnosis, treatment and prognosis of diseases<sup>11</sup>. The ability to produce IFN- $\gamma$  can be used as lymphocyte function<sup>12</sup>. However, the relationship between hyperthyroidism or thyrotoxicosis with immunosenescence has not been given special attention, the material basis of the impact on the immune system also needs to be clarified.

Selecting appropriate aging models and detection indicators are very important for studying aging and exploring anti-aging strategy. The existing aging animal models can reflect the aging characteristics or related diseases in one or some aspects, but cannot comprehensively reflect or detect the physiological changes related to aging<sup>13</sup>, so it is urgent to screen or develop animal models that can fully simulate the characteristics of human aging. Accelerated metabolism by thyroid hormones assumed to accelerated aging. Studies have found that elevated thyroxine levels are associated with aging and shortened lifespan, and the longevity of vertebrate species is usually positively correlated with low metabolic rate and low TH level<sup>14,15</sup>. Despite the length of life is judged in these models, the internal changes of thyroxine affecting aging are rarely mentioned, especially the aging related markers to predict the aging status and the effect of aging intervention. Thyrotoxicosis refers to excessive thyroid hormone in blood circulation caused by any reason, resulting in hyperthyroidism<sup>16</sup>, so exogenous administration of excessive thyroxine can cause thyrotoxicosis. Although many studies have used this model to study the mechanism of hyperthyroidism and the development of hyperthyroidism protective drugs, there is rare discussion on the model simulating aging. We speculate that thyrotoxicosis model is an ideal accelerated aging model, but a deeper understanding of the association between thyrotoxicosis and aging is needed. Our study design allows assessment of the effects of excessive thyroid hormones on immune system and metabolism without autoimmune factors or treatment side effects, especially focus on the influence of metabolites on immune system. On the basis of routine biochemical and hematological indicators, metabonomics was used to analyze the changes of serum endogenous metabolites and fecal metabolites, the mechanisms behind the relationship of thyrotoxicosis and aging will be well explored.

## Results

### Changes of differential blood count and biochemical index

Blood routine test results showed that the total number of white blood cells (WBC), neutrophils (NEUT), lymphocytes (LYMPH), red blood cells (RBC), hemoglobin (HGB), Hematocrit (HCT) and platelets (PLT) were all decreased, which suggested that pancytopenia was induced by excessive thyroxine in mice (Table 1).

Biochemical analysis showed that the levels of Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) and Total triglyceride (TG) were increased significantly after 20 days of continuous intake of overdose thyroxine. The levels of Low-density lipoprotein cholesterol (LDC-C), High-density lipoprotein cholesterol (HDL-C), Total cholesterol (TC), UREA and Creatine (CREA) decreased.

Table 1  
Changes of differential blood count and biochemical index in mice with thyrotoxicosis

		C	T
WBC	10 <sup>9</sup> /L	2.71±0.44	1.97±0.51##↓
NEUT	10 <sup>9</sup> /L	0.39±0.12	0.28±0.09# ↓
LYMPH	10 <sup>9</sup> /L	1.83±0.40	1.24±0.41##↓
RBC	10 <sup>12</sup> /L	9.1±0.9	8.2±0.7# ↓
HGB	g/L	136±12	122±8# ↓
HCT	%	44.7±3.6	41±2.7# ↓
RDW-SD		25.4±1.6	29.1±0.88##↑
RDW-CV		13.1±0.7	14.7±0.6## ↑
PLT	10 <sup>9</sup> /L	1044±95	745±122## ↓
ALT	U/L	36.8±6.6	55.5±10.2## ↑
AST	U/L	81.1±10	106.2±15.3##↑
Glu-G	mmol/L	7.7±1.5	7.0±1.1
TG	mmol/L	1.93±0.44	2.49±0.73#↑
LDC-C	mmol/L	0.21±0.07	0.13±0.03## ↓
HDL-C	mmol/L	1.94±0.42	1.3±0.26## ↓
TC	mmol/L	2.37±0.51	1.62±0.32## ↓
UREA	mmol/L	8.5±1.2	6.1±0.8## ↓
CREA	ummol/L	23.9±1.1	19.3±1.5## ↓

C: normal control mice; T: thyrotoxicosis mice. Compared with normal control mice #  $p<0.05$ , ##  $p<0.01$

## Changes of subsets and function of lymphocytes

As shown in Fig. 1, compared with the normal control group, the percentage of CD3+CD4+ in thyrotoxic mice decreased significantly, while the percentage of CD3+CD8+ increased significantly, so the percentage of CD4+/CD8+ decreased significantly. The percentage of CD3<sup>+</sup>CD4<sup>+</sup> IFN- $\gamma$ <sup>+</sup> was significantly decreased in thyrotoxicosis mice.

## Results of serum LC-MS metabolomics analysis

There were significant differences in OPLS-DA score between thyrotoxicosis group and normal group (Fig. 2a). Based on Human Metabolome Database (HMDB), LC-MS-based metabolomics identified and quantified 821 metabolites in serum, of which 157 were differentially expressed metabolites (DEMs), including 81 upregulated and 76 downregulated (Fig. 2b). KEGG pathway analysis showed that these DEMs were enriched in the pathway of Steroid hormone biosynthesis, Primary bile acid biosynthesis, Bile secretion, Valine, leucine and isoleucine biosynthesis, Arachidonic acid metabolism,

Sphingolipid signaling pathway and so on (Fig. 2c). The classification of DEMs was displayed in Fig. 2d. "Hydroxyl steroid" corresponded to "Steroid hormone biosynthesis", "Phosphosphingolipids" corresponded to "Sphingolipid signaling pathway", the DEMs in these two classes were all upregulated. "Bile acids, alcohols and derivatives" corresponding to "Primary bile acid biosynthesis" and "Bile secretion", there are 11 DEMs in this class, of them 7 were downregulated. "Eicosanoids" corresponded to "Arachidonic acid metabolism", in this class 9 of 11 were downregulated. The DEMs in "Fatty acids and conjugates" and "Linoleic acids and derivatives" classes had ups and down. 7 of 8 DEMs in "Arylsulfates" class were downregulated. The DEMs of "Glycerophosphocholines (GPC)", "Hydroxycinnamic acids and derivatives", "Pregnane steroids", and "Purines and purine derivatives" were all down regulated.

It is noteworthy that significant increases in Thyroxine (T4, FC=12.72,  $p=0.0016$ ) and 3-Iodothyronamine (T3, FC=2.41,  $p=0.0394$ ) were also detected by LC-MS (Fig. 2e), which proved the occurrence of thyrotoxicosis.

## Results of serum lipid metabolomics analysis

Based on Lipidmaps (v2.3) Database, LC-MS-based metabolomics identified and quantified 3333 lipid metabolites in serum, of which 425 were differentially expressed metabolites (DEMs), including 240 upregulated and 185 downregulated (Fig. 2f). The classification of these DEMs were displayed in Fig. 2g. Among them, All Fatty aldehydes, Ceramides, Steroids, Fatty alcohols, Monoradylglycerols, Diradylglycerols, Triradylglycerols, Fatty esters, Glycerophosphoinositolglycans PIM1 and 25 of 29 Fatty Acids and Conjugates were upregulated. The DEMs in Glycerophosphoethanolamines (PE), Glycerophosphoserines (PS), Oxidized glycerophospholipids, Glycerophosphoglycerols (PG), Glycerophosphocholines (PC), Isoprenoids, Fatty amides and Bile acids and derivatives had ups and downs. Hydrocarbons, Eicosanoids, Glycerophosphates (PA) and Glycerophosphoinositols (PI) are mainly downregulated. The DEMs in Steroid conjugates and Docosanoids classes were all downregulated.

## Results of serum GC-MS metabolomics analysis

There were significant differences in OPLS-DA score between thyrotoxicosis group and normal group (Fig. 3a). GC-MS-based metabolomics identified and quantified 885 metabolites in serum, of which 78 were differentially expressed metabolites (DEMs) (Fig. 3b), including 13 upregulated and 67 downregulated. KEGG pathway analysis showed that these DEMs were enriched in the pathway of Protein digestion and absorption, Aminoacyl-tRNA biosynthesis, Biosynthesis of amino acids, Mineral absorption, ABC transporters and so on. These pathways are all related to amino acids. 2 Cholestane steroids (Dihydrocholesterol and Cholesterol) were downregulated.

## Consistency and accuracy of serum MS results

DEMs in serum detected simultaneously by LC-MS and GC-MS included Pyruvic acid, Indoxyl sulfate, Xanthine, Oleic acid, Thymine and Isocitric acid. The results of these DEMs in two methods are consistent, Pyruvic acid, Indoxyl sulfate and Xanthine are all downregulated, while Oleic acid, Thymine and Isocitric acid are all upregulated. The upregulated TGs detected by LC-MS, the downregulated Cholesterol and Creatinine detected by GC-MS, which were in accordance with the results of biochemical test. All the comparisons proved the accuracy of DEMs (Table 2).

Table 2  
The Consistency of DEMs and DEP with different detection methods

Metabolites	Compound ID	Fold Change (FC)		
		LC-MS	GC-MS	Biochemical Test
Pyruvic acid	HMDB0000243	0.36↓	0.63↓	
Indoxyl sulfate	HMDB0000682	0.02↓	0.17↓	
Xanthine	HMDB0000292	0.02↓	0.04↓	
Oleic acid	LMFA01030002	2.46↑		
	HMDB0000207		1.38↑	
Thymine	HMDB0000262	2.11↑	1.31↑	
Isocitric acid	HMDB0000193	2.08↑	1.62↑	
Creatinine	HMDB0000562		0.72↓	0.81↓
Cholesterol	HMDB0000067		0.70↓	0.68↓
TG(18:3/20:0/22:6)	LMGL03016429		2.22↑	1.29↑
TG(12:0/18:3/19:1)	LMGL03013521		6.20↑	
TG(13:0/14:0/22:4)	LMGL03013699		6.74↑	
TG(12:0/17:1/20:3)	LMGL03013408		7.78↑	

## Discussion

### Low immune status and defense ability of thyrotoxicosis

In present study, the syndrome and mechanism of thyrotoxicosis mice were studied by routine detection combined with metabolomics. Blood routine examination showed that excessive thyroxine led to a decrease in the total number of leukocytes, mainly neutrophils and lymphocytes. It also led to anemia and thrombocytopenia. The flow cytometry test showed the decrease of CD4/CD8 ratio and the number of IFN- $\gamma$  + CD4+T cells in thyrotoxicosis mice. The above evidences indicated that the immune function of thyrotoxicosis mice was damaged seriously in present study.

As reported, hyperthyroidism patients may present with single-cell lineage hematological abnormalities such as leucopenia, anemia, thrombocytopenia. The activity of hyperthyroidism (measured at T3 or T4 level) is significantly correlated with anemia and cytopenia<sup>17</sup>. But hyperthyroidism can also lead to pancytopenia<sup>18</sup>. Leukopenia, especially agranulocytosis, may be a complication of hyperthyroidism, which usually leads to severe illness, as well as severe secondary inflammation<sup>19</sup>. The decrease of CD4 / CD8 ratio is one of the markers of T cell "immunosenescence"<sup>9</sup>. The ability to produce IFN- $\gamma$  can be used as lymphocyte function<sup>12</sup>. Th1 CD4 effector T cells can produce large amounts of IFN- $\gamma$  to fight the infection of intracellular pathogens, thereby stimulating and maintaining effective cellular immune responses<sup>20</sup>. In present study, the decrease of CD4/CD8 ratio and the number of IFN- $\gamma$  + CD4+T cells represent the decline of host immune function in thyrotoxicosis mice. Although there is a lack of information on the prevalence and determinants of thyroid function and COVID-19, the British Thyroid Association and the Society for Endocrinology issued a statement emphasizing that patients with thyroid diseases (especially thyrotoxicosis) may have a higher risk of complications, and the American Thyroid Association also recommended that patients with thyroid diseases maintain

social distance and limit their exposure to COVID-19<sup>21</sup>. Actually, autoimmune factors mask the relationship between thyroxine and immunity in human, so the relationship between hyperthyroidism and COVID-19 appears very complex<sup>22</sup>. Here, present study suggested that thyrotoxicosis related immunosenescence might be the cause of aggravation of infection disease, including COVID-19, which also explain the susceptibility and severity of the elderly to respiratory infections caused by decreased resistance. Biochemical test and metabolomics of serum revealed the changes of metabolites partly contributed to immunosenescence.

## Abnormal cholesterol synthesis and metabolism in thyrotoxicosis mice

Biochemical test results and serum metabolomics results together displayed that impairment of lipid metabolism in thyrotoxicosis mice, which reflected by the reduced levels of cholesterol, apolipoproteins and elevated lipid metabolites in serum.

Cholesterol is the basic component of cell membrane, its biosynthetic and regulatory pathways are ubiquitous in various cells, including immune cells, which play an important regulatory role in innate and adaptive immune activities<sup>23</sup>. Although high TC has been advertised as adverse to health, especially in atherosclerosis, studies have shown that low cholesterol is also harmful, hospitalized elderly patients are prone to acquired hypocholesterolemia, which is characterized by low concentrations of all lipoproteins (VLDL, LDL and HDL), which is related to poor prognosis as hypoproteinemia<sup>24</sup>. Plasma cholesterol is a negative acute phase reactant, total cholesterol decreases after surgery and under various pathological conditions, including trauma, sepsis, burn and liver dysfunction, and hypocholesterolemia is associated with in-hospital mortality<sup>25,26,27</sup>. The reason might be cholesterol and apolipoprotein played an important role in pathogen toxin clearance and regulation of inflammatory response<sup>28</sup>. Biochemical test results showed the TC, LDC-C and HDL-C level were decreased significantly in thyrotoxicosis mice. Although the decrease of serum cholesterol level is a known finding in hyperthyroidism<sup>29</sup>, the reason is still unknown. The metabolomic results of present study might give the answer.

Cholesterol homeostasis is regulated by the interaction between endogenous cholesterol synthesis, intestinal diet and bile cholesterol absorption, and bile acid synthesis and excretion. Various plasma markers reflect endogenous cholesterol synthesis (lathosterol, desmosterol, mevalonate, squalene), intestinal cholesterol absorption (sitosterol, campesterol, cholestanol) or bile acid synthesis (7 $\alpha$ -hydroxy-4-cholesten-3-one (C4)) in healthy people and patients<sup>30</sup>. Lanosterol is an intermediate product of cholesterol synthesis<sup>31</sup>. In present study, the endogenous cholesterol synthesis, intestinal cholesterol absorption and bile cholesterol absorption, and bile acid synthesis and excretion were all decreased, the evidences were the downregulation of Lanosterol, Campesterol and bile acids. In addition to Cholesterol, Dihydrocholesterol and 20 $\alpha$ ,22 $\beta$ -Dihydroxycholesterol were also found downregulated. Present study proved that the decrease level of cholesterol attributed to the decrease of synthesis and absorption. And the decrease level of bile acids in present study suggested that the decrease level of Cholesterol was not due to the increasing conversion of cholesterol to bile acids which was accordance with previous study<sup>29</sup>.

Bile acids are cholesterol derived metabolites, which play a recognized role in the digestion and absorption of dietary fat<sup>32</sup>. Bile acids are a physiological factor required for nutrient absorption, distribution, metabolism and excretion. They are also nutrient sensor and metabolic regulator<sup>33</sup>. Bile acids also have important immunomodulatory effects<sup>34</sup>. The primary bile acids produced by the liver are metabolized into secondary bile acids under the action of intestinal microbes, they play a role in maintaining intestinal barrier and preventing intestinal pathogens from colonization. These primary and secondary bile acids play a beneficial role in maintaining innate immunity by acting on their receptors at the

interface of the host immune system<sup>35</sup>. Taurochenodeoxycholic acid has been found to enhance immunity by increasing CD4+/CD8+ value in peripheral blood in mice<sup>36</sup>. Chenodeoxycholic acid can inhibit the lipotoxicity of cardiomyopathy<sup>37</sup>. Previous study showed that the T3 dose dependently decreased the formation of cholic acid and chenodeoxycholic acid by inhibiting the expression of CYP7A1 and Cyp8b1 human liver cell lines<sup>38</sup>. In present study, several of bile acids were downregulated, including 3a,7a-Dihydroxy-5b-cholestan-26-al, Chenodeoxycholic acid, Chenodeoxycholic acid 3-glucuronide, Alpha-Muricholic acid, Deoxycholic acid, Taurochenodesoxycholic acid, Dihomodeoxycholic acid and Lithocholic acid. Especially Chenodeoxycholic acid and Deoxycholic acid reduced to undetectable levels. The downregulation of these bile acids seriously affects the metabolism of the lipids and drugs<sup>39</sup>.

Vitamin D is another product of cholesterol metabolism. In addition to the classical effects related to mineral homeostasis, vitamin D plays a new role in cell proliferation and differentiation, regulation of innate and adaptive immune system, prevention of cardiovascular and neurodegenerative diseases, and even anti-aging<sup>40</sup>. A recent study found that the serum 25-OHvit D levels in hyperthyroidism patient with hypercalcemia were lower than the normal range, and Vitamin D3 adjuvant therapy can improve thyroid related antibody level, thyroid function and bone metabolism in patients with hyperthyroidism complicated with hypercalcemia<sup>41</sup>. Early study showed that aging could reduce the ability of skin to produce previtamin D3 more than two times<sup>42</sup>. Many aging related diseases are associated with decreased vitamin D3 levels, and Vitamin D deficiency remains a global public health problem<sup>43</sup>. In present study, (23E)-26,26,26,27,27,27-hexafluoro-1alpha,25-dihydroxy-23,24-didehydrovitamin D3 and (22E)-26,26,26,27,27,27-hexafluoro-1alpha,25-dihydroxy-22,23-didehydrovitamin D3 were found downregulated in thyrotoxicosis mice. As reported, the potency of 26,26,26,27,27,27-hexafluoro-1 alpha,25-dihydroxyvitamin D3 (26,27-F6-1,25(OH)2D3) to enhance bone calcium (Ca) mobilization was higher than that of 1 alpha,25-dihydroxyvitamin D3<sup>44</sup>, its activity is about 10 times that of 1,25-dihydroxyvitamin D3 (1,25(OH)2D3)<sup>45</sup>. It was the first time to detected the decrease of 26,27-F6-1,25(OH)2D3 in thyrotoxicosis, and which suggested that the 26,27-F6-1,25(OH)2D3 might play important role in protection body from toxicity caused by excessive thyroid. And direct supplementation or measures that can improve VD3 level especially 26,27-F6-1,25(OH)2D3 may have protective effect.

The decrease level of bile acids proved that the decrease level of Cholesterol was not due to the increasing conversion of cholesterol to bile acids. Alternatively, it might due to the increasing conversion to glucocorticoids. Conversely to the downregulation of bile acids, another cholesterol metabolites glucocorticoids were upregulated in thyrotoxicosis mice, which might partly contribute to the decline of Cholesterol. They were all Hydroxysteroids, including Tetrahydrocorticosterone, 11b,17a,21-Trihydroxypreg-nenolone, Cortolone, 3a,21-Dihydroxy-5b-pregnane-11,20-dione, 18-Hydroxycorticosterone, Cortisol, 11-Dehydrocorticosterone, Dihydrocortisol, Corticosterone and Cortol (Table 3). Their upregulations indicated the increase of endogenic glucocorticoids (eGCs) in plasma which was in line with previous study<sup>46</sup>. As reported, increased hypothalamic- pituitary- adrenal (HPA) axis activity associated with cushing's syndrome, hyperthyroidism and aging<sup>47</sup>. GC is the most common cause of secondary osteoporosis and the main cause of non-traumatic osteonecrosis<sup>48</sup>. It is well known that GCs have immunosuppressive effect, which inhibits phagocytosis of macrophages and causes lymphocytic lysis, especially the decrease of helper T cells (Th)<sup>49</sup>. And CD4 + T cells are highly sensitive to GC induced apoptosis<sup>50</sup>. eGCs levels increase with age and can accelerate aging processes in vertebrate species<sup>51,52</sup>. Research also indicated that stress-induced GCs might play causal role for aging and age-related disorders<sup>53</sup>. Aging and chronic stress together lead to abnormal HPA axis activation, leading to the increase of peripheral GC level, consequently accelerate cell aging and premature immunosenescence, including the reduction of primitive T cells, poor immune response to neoantigens, cell-mediated immune reduction, and thymus degeneration, resulting in an increase in the incidence of diseases related to immunosenescence, including tumors and COVID-19<sup>54,55</sup>. So, the increase of GCs is both the result and the cause of aging. And in present study the upregulated eGCs in

thyrotoxicosis mice are not only the reason for the decline of immunity, but also the evidence that the model simulates aging.

In general, the changes in cholesterol synthesis and metabolism were mainly manifested by the decreasing of synthesis and bile acids mediated absorption, as well as the increasing conversion to glucocorticoids. The decrease of cholesterol, bile acids and VD, as well as excessive glucocorticoids resulting in immune decline, which are closely related to the aging. The cholesterol related differential metabolites were integrated into Table 3.

Table 3  
The changes of cholesterol and derivatives

Metabolites	Compound ID	Formula	FC	P-value
<b>Cholesterol synthesis</b>				
Lanosterol	HMDB0001251	C30H50O	0.69	0.0019
Campesterol	HMDB0002869	C28H48O	0.34	2.9E-07
Dihydrocholesterol	HMDB0001569	C27H48O	0.21	0.0001
20a,22b-Dihydroxycholesterol	HMDB0006763	C27H46O3	0.31	0.0087
Cholesterol	HMDB0000067	C27H46O	0.70	0.0122
<b>Bile acids and derivatives</b>				
Chenodeoxycholic acid	HMDB0000518	C24H40O4	1.2E-08	3.6E-06
Chenodeoxycholic acid 3-glucuronide	LMST05010022	C30H48O10	0.87	0.0091
Deoxycholic acid	HMDB0000626	C24H40O4	7.3E-08	0.0006
3a,7a-Dihydroxy-5b-cholestan-26-al	HMDB0006894	C27H46O3	0.18	0.0005
Taurochenodesoxycholic acid	HMDB0000951	C26H45NO6S	0.006	0.0008
Alpha-Muricholic acid	HMDB0000506	C24H40O5	0.05	0.0027
Dihomodeoxycholic acid	LMST04020031	C26H44O4	0.48	0.0123
Lithocholic acid	LMST04010003	C24H40O3	0.69	0.0136
<b>vitamin D</b>				
25-hydroxyvitamin D2 25-(beta-glucuronide)	LMST05010021	C34H52O8	0.84	0.0462
(23E)-26,26,26,27,27,27-hexafluoro-1alpha,25-dihydroxy-23,24-didehydrovitamin D3	LMST03020083	C27H36F6O3	0.42	0.0021
(22E)-26,26,26,27,27,27-hexafluoro-1alpha,25-dihydroxy-22,23-didehydrovitamin D3	LMST03020082	C27H36F6O3	0.44	0.0058
<b>Hydroxysteroids</b>				
Tetrahydrocorticosterone	HMDB0000268	C21H34O4	2.28	0.0318
11b,17a,21-Trihydroxypreg-nenolone	HMDB0006760	C21H32O5	2.46	0.0446
Cortolone	HMDB0003128	C21H34O5	3.67	0.0052
3a,21-Dihydroxy-5b-pregnane-11,20-dione	HMDB0006755	C21H32O4	3.90	0.0042
18-Hydroxycorticosterone	HMDB0000319	C21H30O5	6.55	0.0263
Cortisol	HMDB0000063	C21H30O5	14.26	9.8E-07
11-Dehydrocorticosterone	HMDB0004029	C21H28O4	14.68	7.6E-06

Metabolites	Compound ID	Formula	FC	P-value
Dihydrocortisol	HMDB0003259	C21H32O5	146.11	0.0024
Corticosterone	HMDB0001547	C21H30O4	209.90	0.0335
Cortol	HMDB0003180	C21H36O5	382.37	0.0003
11-Dehydrocorticosterone	LMST02030192	C21H28O4	1.86	0.0245
Corticosterone	LMST02030186	C21H30O4	2.56	0.0376

## Lipotoxicity in thyrotoxicosis mice

Early study found that T3 or T4 may directly stimulate the lipolysis which is characterized by plasma high fatty acids <sup>56</sup>, however, there is no discussion about the relationship between high plasma fatty acids and lipotoxicity in hyperthyroidism. In present study, the lipids DEMs such triglycerides (TGs), Fatty acids and Sphingolipids were upregulated, which indicated lipotoxicity in thyrotoxicosis mice.

It is generally believed that the increase in blood lipids is due to the use of antithyroid drugs <sup>57</sup>. However, this study found that excessive thyroxine itself also leads to the increase of TGs level in serum. Combined with the upregulation of Monoradylglycerols, Diradylglycerols and large number of upregulated Fatty acids, a complete map of TGs metabolism in thyrotoxicosis model was displayed, that is the increase of fatty acids attributed to thyroxine mobilized the decomposition of adipose tissue, resulting in the increase of free fatty acids level in blood, with the increase of Monoradylglycerols (MGs), Diradylglycerols (DGs) led to the increase of TGs synthesis, at the same time, because of the decrease of lipoprotein, TG couldn't be transported in time, leading to the high level of TGs in serum <sup>58</sup>.

As well known, TGs are highly correlated with aging and age-related physiological dysfunction <sup>59</sup>. Various intermediates of fatty acid metabolism have been shown to cause cell stress and toxicity (lipotoxicity) in adipocytes and other related cell types (including cardiomyocytes, hepatocytes and immune cells), such as Sphingolipid, Ceramide and Diacylglycerol, which were also upregulated in present study <sup>60</sup>. Cells treated with sphingosine can rapidly induce mitochondrial membrane potential loss, mitochondria release cytochrome c and apoptotic cell death <sup>61</sup>. Sphingolipids implicated in the pathophysiology of cardiovascular disease, and inhibition of sphingolipid synthesis could attenuate cardiomyopathic symptoms <sup>62</sup>. Sphingolipids, including ceramide, play a role in aging and are also markers of aging. Many studies have shown that lowering ceramide concentration may delay or improve the symptoms of aging human aging <sup>59</sup>. Here, the upregulated Sphingolipids are another evidence that thyrotoxicosis mimics aging.

It was found that plasma long-chain free fatty acids are inversely correlated with longevity <sup>63</sup>. And long-chain fatty acids also affect immune cells. For example, it was found that a  $\omega$ -6 polyunsaturated fatty acids (PUFAs) Linoleic acid (18:2) destroyed mitochondrial function, caused more oxidative damage than other free fatty acids (such as Palmitic acid), which mediated the selective loss (death) of CD4 (+) T lymphocytes in the liver, accelerated the occurrence of cancer <sup>64</sup>. Linoleic acid could also decrease the function of immune cells by inhibited IFN- $\gamma$  production in CD4<sup>+</sup> T cells <sup>65</sup>. The severity of ICU patients with COVID-19 was associated with high levels of free fatty acid <sup>66</sup>. Mice given linoleic acid (LA) (C18:2) developed leukopenia, lymphopenia, lymphocyte damage, relative thrombocytopenia, hypercytokinemia, elevated alanine aminotransferase levels, hypoalbuminemia, hypocalcemia, shock, and renal failure, resembling lethal COVID-19 <sup>67</sup>. In present study, Linoleic acid was also upregulated which might be the cause of loss and dysfunction of CD4 T cells.

On the contrary,  $\omega$ -3 fatty acids have potential use in COVID-19 treatment because they have their antioxidant and anti-inflammatory effects, as well as the ability of regulating platelet homeostasis and the risk of thrombosis<sup>68</sup>. A pilot study in 100 patients suggested that  $\omega$ -3 fatty acids have a tendency to reduce the incidence rate and mortality of COVID-19 infection<sup>69</sup>. As we all know, intake  $\omega$ -3 fatty acids can have beneficial health effects on many biological processes, such as improving immune status, protecting against infection and allergies, enhancing cognitive ability, optimizing neuromuscular function and reducing muscle loss<sup>70,71</sup>.  $\omega$ -3 fatty acids regulate lipid metabolism, contribute to fatty acid oxidation and inhibit fat production, and lead to good lipid distribution and adipocyte metabolism<sup>72</sup>.  $\omega$ -3 fatty acids improve body composition by reducing cortisol levels<sup>73</sup>, which suggested that  $\omega$ -3 fatty acids could resist the adverse effects of cortisol. Coincidentally, the level of cortisol were upregulated, while the  $\omega$ -3 fatty acids and their metabolites were found downregulated in thyrotoxicosis mice in present study, including Docosapentaenoic acid (22n-3) (DPA), Maresin 1 and 17,18-EpETE. So, the less of  $\omega$ -3 fatty acids might also be the cause of harmful effect of eGCs in present study. The effects of  $\omega$ -3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been widely studied. DPA is the metabolic intermediate of EPA and DHA, less is known about DPA, however, available evidence suggests that DPA may also be superior to the health benefits of EPA and DHA<sup>74</sup>. DPA has anti-inflammatory effects and can improve cardiovascular and metabolic diseases, and plasma DPA level was inversely associated with total mortality in older people<sup>75</sup>. In addition, DPA is particularly beneficial to the neuroprotection and early life development of the elderly<sup>76</sup>. DPA is also a precursor of docosanoids, such as Maresin 1, which was found downregulated in thyrotoxicosis mice in present study. Maresin 1 plays an important role in the remission of acute inflammation and organ protection by enhancing the phagocytosis of macrophages, which is beneficial to maintain host defense ability, homeostasis and wound healing<sup>77</sup>. In addition, 17,18-epoxyeicosatetraenoic acid (17,18-EpETE) was also found downregulated, it is a lipid metabolite endogenously generated from EPA which exhibits anti-allergic and anti-inflammatory properties<sup>78</sup>.

The upregulations of Glycerolipids, Sphingolipids, Fatty Acids and the downregulation of Docosanoids in present study were listed in Table 4.

Table 4  
The changes of lipid metabolites

Metabolites	Compound ID	Formula	FC	P-value
<b>Glycerolipids</b>				
DG(18:2(9Z,12Z)/18:2(9Z,12Z)/0:0)	HMDB0007248	C39H68O5	2.18	0.0411
DG(16:0/18:2(9Z,12Z)/0:0)	HMDB0007103	C37H68O5	3.85	0.0136
DG(16:1(9Z)/18:2(9Z,12Z)/0:0)	HMDB0007132	C37H66O5	10.80	0.0211
DG(18:1(9Z)/18:2(9Z,12Z)/0:0)[iso2]	LMGL02010056	C39H70O5	3.90	0.0009
1-O-(2R-hydroxy-hexadecyl)-sn-glycerol	LMGL01020063	C19H40O4	1.93	0.0298
TG(18:3(6Z,9Z,12Z)/20:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z)) [iso6]	LMGL03016429	C63H104O6	2.22	0.0210
TG(12:0/18:3(9Z,12Z,15Z)/19:1(9Z))[iso6]	LMGL03013521	C52H92O6	6.20	0.0006
TG(13:0/14:0/22:4(7Z,10Z,13Z,16Z))[iso6]	LMGL03013699	C52H92O6	6.74	0.0010
TG(12:0/17:1(9Z)/20:3(8Z,11Z,14Z))[iso6]	LMGL03013408	C52H92O6	7.78	0.0008
<b>Sphingolipids</b>				
GM4(d18:1/16:0)	LMSP0601AA01	C51H94N2O16	1.48	0.0270
CerP(d18:1/18:0)	LMSP02050004	C36H72NO6P	1.73	0.0240
Cer(d18:0/14:0)	LMSP02020016	C32H65NO3	1.79	0.0337
GlcCer(d15:2(4E,6E)/20:0)	LMSP0501AA59	C41H77NO8	1.51	0.0595
GlcCer(d15:2(4E,6E)/22:0)	LMSP0501AA60	C43H81NO8	1.82	0.0219
N-(hexadecanoyl)-deoxysphing-4-enine-1-sulfonate	LMSP00000002	C34H67NO5S	1.70	0.0106
N-(tetradecanoyl)-deoxysphing-4-enine-1-sulfonate	LMSP00000001	C32H63NO5S	1.98	0.0203
SM(d16:1/20:0)	LMSP03010052	C41H83N2O6P	1.80	0.0727
SM(d18:2/24:0)	LMSP03010081	C47H93N2O6P	5.65	0.0109
SM(d18:1/24:1(15Z))	LMSP03010007	C47H93N2O6P	6.57	0.0109
LysoSM(d18:1)	HMDB0006482	C23H50N2O5P	2.15	0.0426
Sphingosine 1-phosphate	HMDB0000277	C18H38NO5P	2.45	0.0003
Sphinganine 1-phosphate	HMDB0001383	C18H40NO5P	3.69	0.0009
SM(d18:1/24:1(15Z))	HMDB0012107	C47H93N2O6P	7.29	3E-05
<b>Fatty Acids and Conjugates</b>				
Hydroxyphthioceranic acid (C40)	LMFA01020326	C40H80O3	1.25	0.0256
6-bromo-tricoso-5E,9Z-dienoic acid	LMFA01090101	C23H41BrO2	1.28	0.0116
29:2(5Z,9Z)(6Br)	LMFA01030891	C29H53BrO2	1.29	0.0004
Linoleic acid	LMFA01030120	C18H32O2	1.89	0.0083

Metabolites	Compound ID	Formula	FC	P-value
Oleic acid	LMFA01030002	C18H34O2	2.46	0.0077
10-hydroxy-16-oxo-hexadecanoic acid	LMFA01170060	C16H30O4	1.46	0.0246
trans-9-palmitoleic acid	LMFA01030057	C16H30O2	2.18	0.0036
13-hexadecenoic acid	LMFA01030263	C16H30O2	3.01	0.0026
6Z,9Z-hexadecadienoic acid	LMFA01030273	C16H28O2	3.05	0.0119
Tetradecanedioic acid	LMFA01170018	C14H26O4	2.90	0.0010
Tetranor-8-NO2-CLA	LMFA01120009	C14H23NO4	1.4E+08	0.0230
2-methyl-dodecanedioic acid	LMFA01170010	C13H24O4	1.57	0.0130
11R-hydroxy-dodecanoic acid	LMFA01050253	C12H24O3	1.38	0.0301
11-hydroxy-dodecanoic acid	LMFA01050165	C12H24O3	1.39	0.0302
9-hydroxy-dodecanoic acid	LMFA01050167	C12H24O3	1.43	0.0105
xi-5-Hydroxydodecanoic acid	LMFA01050529	C12H24O3	1.47	0.0162
4-hydroxy lauric acid	LMFA01050038	C12H24O3	1.54	0.0048
3-hydroxy-dodecanedioic acid	LMFA01160025	C12H22O5	1.88	0.0248
Oleic acid	HMDB0000207	C18H34O2	1.39	0.0027
Palmitoleic acid	HMDB0003229	C16H30O2	2.81	0.0096
Palmitelaidic acid	HMDB0012328	C16H30O2	2.10	0.0140
Beta-hydroxymyristic acid	HMDB0061656	C14H28O3	1.72	0.0363
<b>Docosanoids and metabolites</b>				
DPA	LMFA04000044	C22H34O2	6E-08	0.0448
Docosapentaenoic acid (22n-3)	HMDB0006528	C22H34O2	6E-08	0.0020
Maresin 1	LMFA04050001	C22H32O4	0.78	0.0196
17,18-EpETE	HMDB0010212	C20H30O3	0.12	0.0065

## Perspectives and summary: the relationship of thyrotoxicosis with aging

In this study, routine examination combined with metabonomics technology were used to make a comprehensive research report on thyrotoxicosis. Bioinformatics combined with a large number of literature research was used to deeply analyze the serum metabonomics. A variety of test results were mutually verified, and the relationship between thyrotoxicosis and aging was well established. The results were summarized in Fig. 4.

Serum metabolomics analysis showed that the metabolism of thyrotoxicosis mice was disordered, especially the synthesis and metabolism of cholesterol and lipids. The decrease in cholesterol synthesis and absorption led to a

decrease in serum cholesterol, which consequently resulted in a decline in the levels of cholesterol derived VD and bile acids. The lack of VD led to osteoporosis and the decline of immunity. The decrease in bile acids not only caused disorder of lipid metabolism, but also contributed to decline in innate immunity. In contrast, another type of cholesterol derivative GCs increased significantly, which can cause osteoporosis, immunosenescence. Despite that VD and bile acids were downregulated while the GCs were upregulated, the results caused by their changes are indeed consistent, which are harmful to the body and relate to aging.

This study also showed that thyrotoxicosis model might be an appropriate model to study lipotoxicity, because a variety of lipotoxicity related metabolites were up-regulated, including Glycerolipids, Sphingolipids and Fatty Acids. Lipotoxicity is mainly manifested in its damage to the mitochondrial membrane, acting on immune cells leading to immunosenescence, and acting on organs causing organ senescence. Furthermore, GCs are also Lipids and lipid-like molecules, and the harmful effects of excessive GCs can also be classified as lipotoxicity. On the contrary, the Docosanoids were downregulated, but correspondingly, their decrease also led to the decline of homeostasis regulation ability and defense ability.

In summary, as shown in Fig. 4, the increase of harmful lipids metabolites and the decrease of protective lipids metabolites eventually lead to the aging feather of thyrotoxicosis mice. In present study, the symptoms of aging were manifested as immunosenescence. Present study provided evidence that thyrotoxicosis model is an aging model, which can be used to study the mechanism of aging and anti-aging drugs, even to explore disease prevention measures, like COVID-19. Studies have confirmed that COVID-19 is an acute aging disease<sup>4</sup>, age and age-related diseases are the main risk factors leading to severe disease and COVID-19 death, but the reason for this age dependence is not clear<sup>79</sup>. Present study provides some clues, the decline of CD4/ CD8 ratio and IFN- $\gamma$  production capacity, granulocytopenia indicated that the immune function and the defense ability of thyrotoxicosis mice was damaged seriously, the high levels of free fatty acid and eGCs were indicated lipotoxicity in thyrotoxicosis mic, all above factors was related with the severity of COVID-19 patients. Candidates that can improve lipotoxicity of thyrotoxicosis may have potential value in the development of anti-aging drugs, whether they have anti-thyroxine function or not.

The combination of metabonomics and conventional detection methods to study the toxic effects of excess thyroxine provide more information for the thyrotoxicosis model to simulate aging, mainly manifested as immunosenescence in present study, whereas the differential metabolites are also rooted in the generated organs or tissues, and also represent the aging lesions of organs. The organ changes might provide more informations for exploring the similarity of thyrotoxicosis and aging, and the proteomics of serum may provide more convenient markers for judgement of aging.

The subsequent use of system biology to study serum and organ changes will make the evidence more complete for thyrotoxicosis simulating aging.

## Methods

### Animals and reagents

4-6 weeks old healthy female KM mice (SPF grade) with body weights of 16-18 g was purchased from Ji'nan Pengyue Laboratory Animal Breeding Co., Ltd (Shandong, China). The mice were housed in a clean room at a temperature of  $23\pm 2^{\circ}\text{C}$  and a humidity of  $50\pm 5\%$  with a 12 h alternating light and dark cycle. They were permitted free access to food and water. All animal experiments were performed according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of Shandong Province, China. The thyroxine tablets were purchased from Shandong Renhe Pharmaceutical Co., Ltd (China). Rat Anti-Mouse CD3 antibody, Rat Anti-Mouse CD4 antibody, Rat Anti-Mouse IFN- $\gamma$ , Leukocyte Activation Cocktail, with BD GolgiPlug (Cat.No.550583) were purchased from BD PharmingenTM. All chemicals and solvents were analytical or HPLC grade

Water, methanol, acetonitrile, formic acid, pyridine, n-hexane, methoxylamine hydrochloride, N, O-Bis (trimethylsilyl) trifluoroacetamide (BSTFA) with 1% chlorosilane were purchased from CNW Technologies GmbH (Germany), L-2 chlorophenylalanine was from Shanghai Hengchuang Bio technology Co Ltd. (China).

## Thyrotoxicosis model

24 mice were randomly divided into 2 groups (n = 12 per group): control group (C), model group (T). Intra-gastric administration of thyroxine tablets suspension (320mg / kg), once a day, for 20 days. The body weight was measured before and at the end of the model. At the end of the experiment, fasting the night before dissection, blood was taken from the main abdominal vein after anesthesia with 60mg·kg<sup>-1</sup> pentobarbital sodium, and 6 mice in each group were for routine blood test and flow cytometry. 6 mice in each group were for biochemical analysis and metabonomics detection. About 1ml blood was injected to gel separation tube and the serum was separated after centrifugation of 1000g. 100ul serum was taken out and diluted 4 times for biochemical detection. The remaining serum was cryopreserved at - 80 °C for proteomics and metabonomics detection.

## Routine blood tests

EDTAK2 anticoagulant whole blood was taken for routine blood test. Routine blood tests were carried by Sysmex XN-1000v [B1] automated hematology analyzer (SYSMEX Co., Ltd., Japan).

## Detection of CD4<sup>+</sup>, CD8<sup>+</sup> T lymphocytes

The positive rates of CD4<sup>+</sup>, CD8<sup>+</sup> T lymphocyte subsets were analyzed by flow cytometry. Briefly, 3 mL of red blood cell lysis buffer was added to each sample to completely lyse red blood cells. Then the samples were washed and re-suspended with DMEM to 1 × 10<sup>6</sup> cells/mL. Cells were transferred to 48-well plates with 100 μL volume of each well, 2 μL of cell activation cocktail (BD biosciences) and 1 μL of BrefeldinA (BD biosciences) were added to each sample and incubated at 37 °C for 6 h. After washed and re-suspended with PBS, samples were incubated with specific fluorescent antibodies (BD biosciences) of PC5.5 conjugated anti-mouse CD3 antibody (0.5 μl/sample), FITC conjugated anti-mouse CD4 antibody (0.2 μl/sample), and APC-A750 conjugated anti-mouse CD8 antibody (0.5 μl/sample) for 30 min at room temperature in dark according to the manufacturer's guidelines. All samples were stained in triplicate. The samples were analyzed by the CytoFLEX flow cytometer (Beckman Coulter Life Sciences), and the data were analyzed by the CytExpert software (Beckman Coulter Life Sciences).

## Detection of CD4<sup>+</sup>IFN-γ<sup>+</sup> T lymphocytes

To further determine the levels of CD4<sup>+</sup>IFN-γ<sup>+</sup> T lymphocyte subsets, cells treated in the previous step were fixed with 500 μL of 4% FA for 20 min in dark, ruptured with 1 mL of Permeabilization Wash Buffer (BD biosciences). Then the cells were stained with phycoerythrin (PE) conjugated anti-mouse IFN-γ antibody (1.25 μg/sample) for 30 min. All samples were stained in triplicate. The samples were analyzed by the CytoFLEX flow cytometer (Beckman Coulter Life Sciences), and the data were analyzed by the CytExpert software (Beckman Coulter Life Sciences).

## Biochemical Analysis

Serum biochemical analysis were detected by BS-800 automatic biochemistry analyzer (Shenzhen Mindray Bio-Medical Electronics CO., Ltd., China).

## Measurement of serum metabolome using LC-MS

120  $\mu$ L serum of each sample added to a 1.5 mL Eppendorf tube with internal standard, and then ice-cold mixture of methanol and acetonitrile (2:1 v/v) was added to precipitate protein. The tube was centrifuged and the final supernatant was filtered through 0.22  $\mu$ m microfilters and transferred to LC vials. QC samples were prepared by mixing aliquots of the all 18 samples to be a pooled sample. The vials were stored at 80 °C until LC-MS analysis. An ACQUITY UHPLC system (Waters Corporation, Milford, USA) coupled to an AB SCIEX Triple TOF 5600 System (AB SCIEX, Framingham, MA) was used to analyze the metabolic profiles in both ESI positive and ESI negative ion modes. An ACQUITY UPLC BEH C18 column (100 mm  $\times$  2.1mm, 1.7  $\mu$ m) were employed in both positive and negative modes. The QCs were injected at regular intervals (every 6 samples) throughout the analytical run to provide a set of data from which repeatability can be assessed. Metabolites were identified by progenesis QI (Waters Corporation, Milford, USA) Data Processing Software, based on public databases such as Human Metabolome Database (<http://www.hmdb.ca/>) and LIPID MAPS Structure Database (<http://www.lipidmaps.org/>).

## Measurement of serum metabolome using GC-MS

80  $\mu$ L of sample was added to a 1.5 mL Eppendorf tube with internal standard. Subsequently, ice-cold mixture of methanol and acetonitrile (2/1, v/v) was added centrifuged. QC sample was prepared by mixing aliquots of the all samples to be a pooled sample. An aliquot of the 150  $\mu$ L supernatant was transferred to a glass sampling vial for vacuum dry at room temperature. And 80  $\mu$ L of 15 mg/mL methoxyamine hydrochloride in pyridine was subsequently added. The resultant mixture was vortexed vigorously for 2 min and incubated at 37 °C for 90 min. 80  $\mu$ L of BSTFA (with 1% TMCS) and 20  $\mu$ L n hexane was added into the mixture, which was vortexed vigorously for 2 min and then derivatized at 70 °C for 60 min. The samples were placed at ambient temperature for 30 min before GC MS analysis. The derivatized samples were analyzed on an Agilent 7890B gas chromatography system coupled to an Agilent 5977A MSD system (Agilent Technologies Inc., CA, USA). ADB-5MS fused-silica capillary column (30m $\times$ 0.25mm $\times$ 0.25 $\mu$ m, Agilent J&W Scientific, Folsom, CA, USA) was utilized to separate the derivatives. Helium (>99.999%) was used as the carrier gas at a constant flow rate of 1 mL/ min through the column. The QCs were injected at regular intervals (every 6 samples) throughout the analytical run to provide a set of data from which repeatability could be assessed. Metabolites were identified by progenesis QI (Waters Corporation, Milford, USA) Data Processing Software, based on public databases such as Human Metabolome Database.

## Data and Statistical Analysis

All experimental data obtained from rats were expressed as mean  $\pm$  SD. A one-way repeated measure analysis of variance (ANOVA) and a log-rank test were used to determine the significance of the differences in differential blood count, biochemical index and subsets and function of lymphocytes, respectively.

For LC-MS and GC-MS, differential expressed metabolites (DEMs) were selected on the basis of the combination of a statistically significant threshold of variable influence on projection (VIP) values obtained from the (orthogonal) partial least-squares-discriminant analysis (OPLS-DA) model and p values from a two tailed Student's t test on the normalized peak areas from different groups, where metabolites with VIP values larger than 1.0 and p values less than 0.05 were considered as differential metabolites.

# Declarations

## Acknowledgment

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## Availability of data materials

The data is available with the corresponding author and will be provided upon the legitimate request.

## Conflict of interest

The authors declare no conflict of interest.

## Ethics statement

This study was carried out according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and was approved by the Animal Care and Use Committee of Shandong Province, China.

## Author contributions

Guimin Zhang conceived the project and designed the experiments. Qin Feng, Wenkai Xia, Guoxin Dai, Jingang Lv, Jian Yang conducted the experiments. Qin Feng performed proteomic analysis and wrote the paper.

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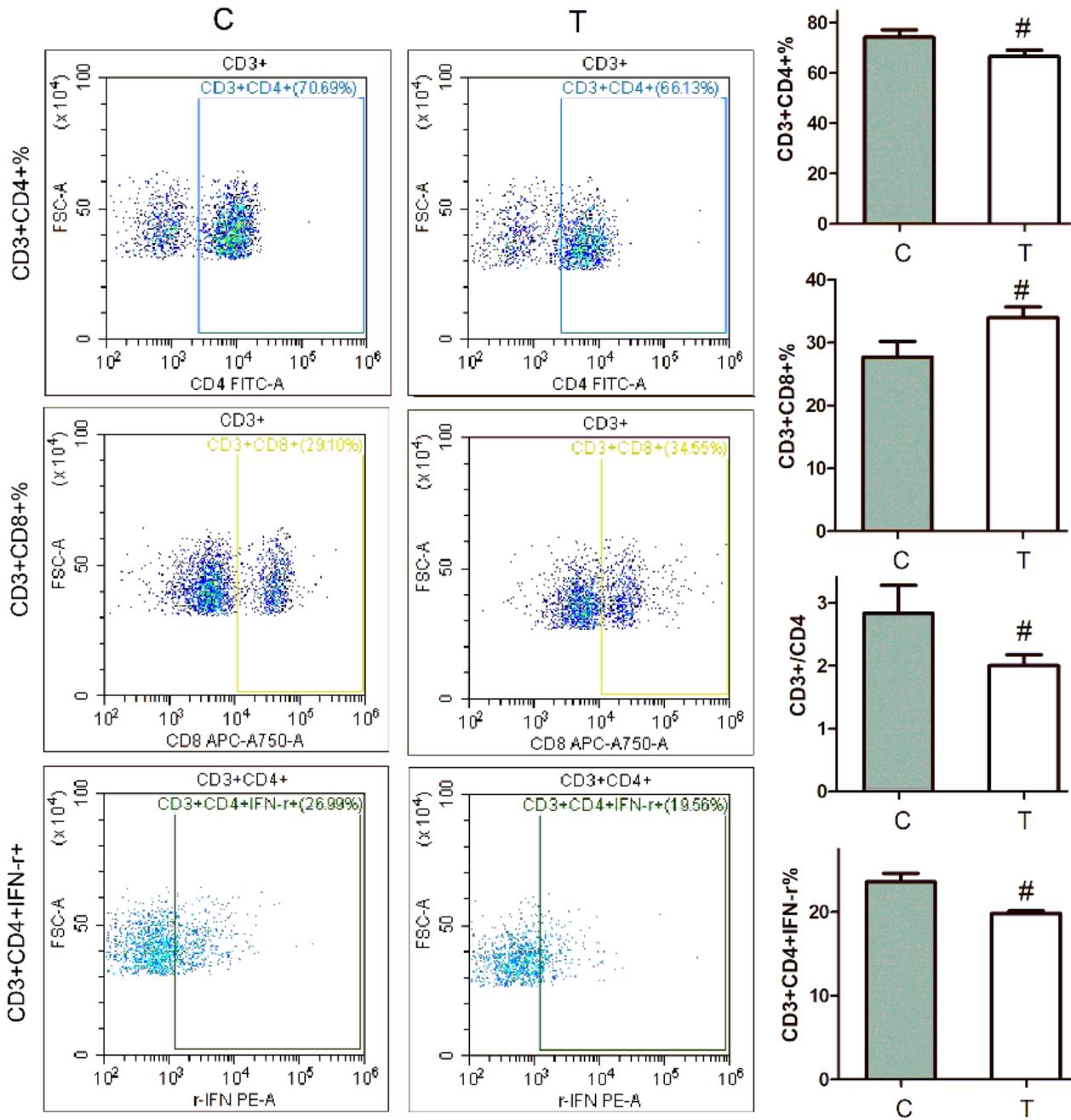
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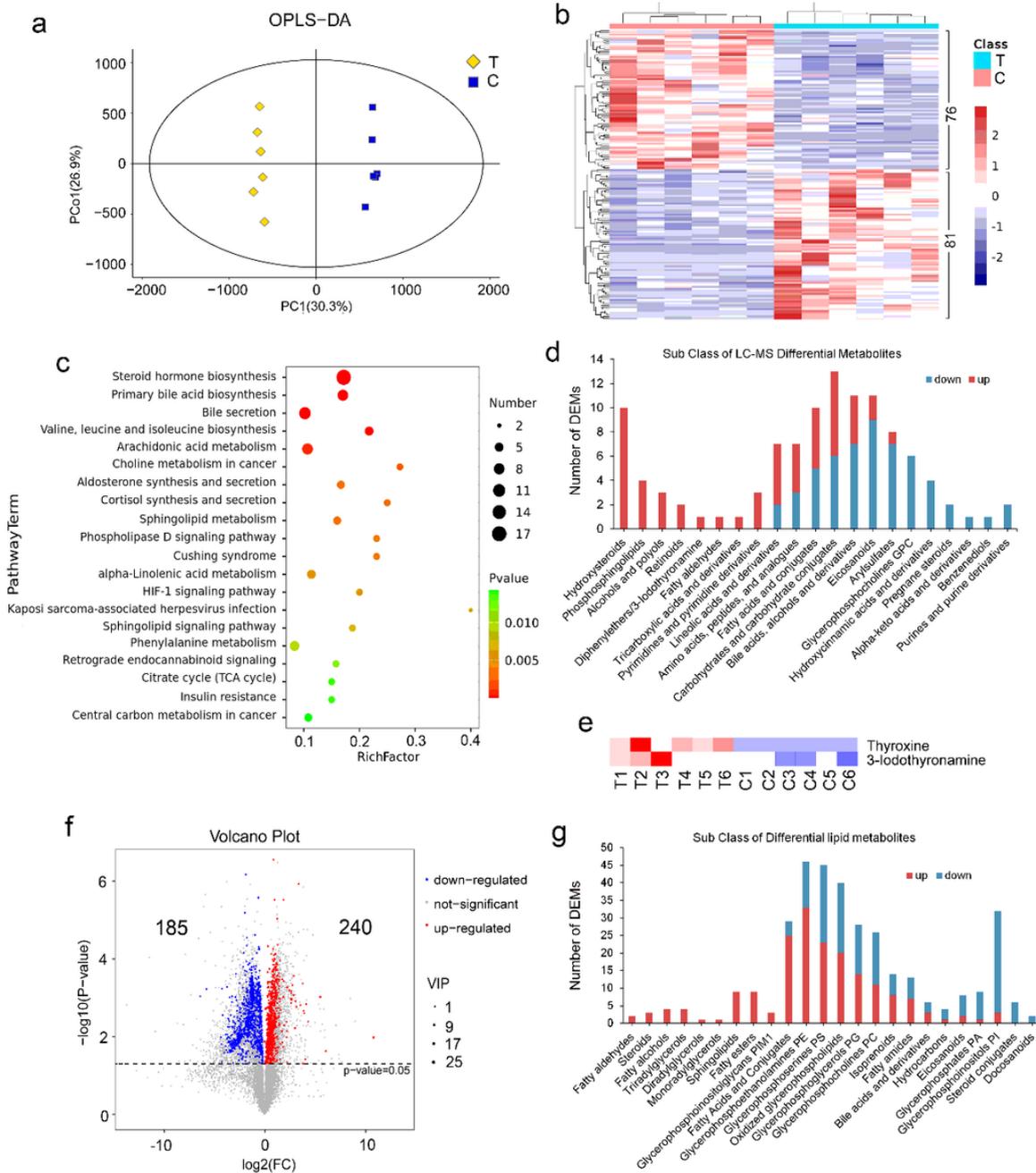
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## Figures



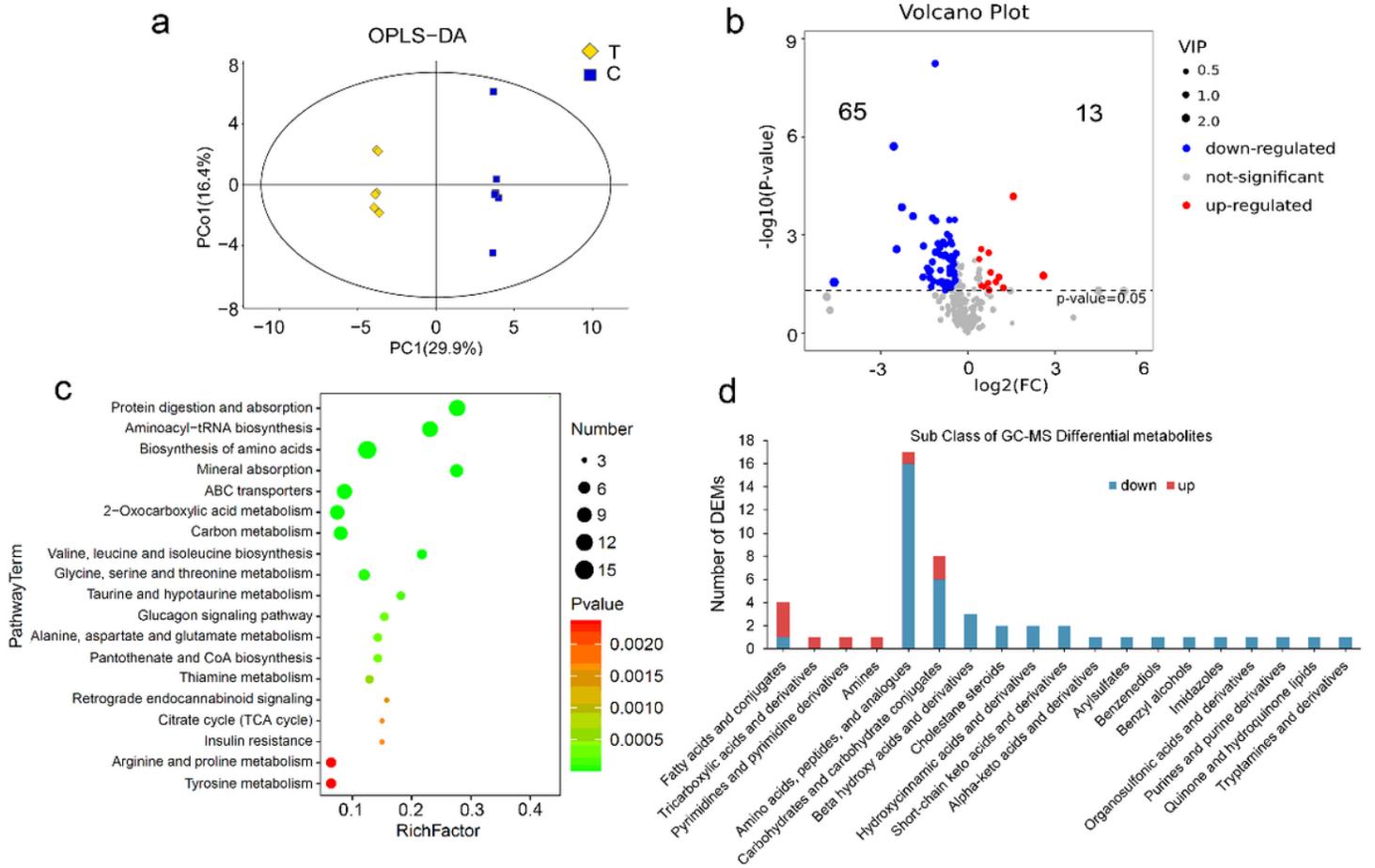
**Figure 1**

**Changes of subsets and function of lymphocytes.** Compared with the normal control group, the percentage of CD3+CD4+ in thyrotoxicosis mice decreased significantly, while the percentage of CD3+CD8+ increased significantly, so the percentage of CD4+/CD8+ decreased significantly. The percentage of CD3+CD4+ IFN-r+ was significantly decreased in thyrotoxicosis mice. C: normal control group; T: thyrotoxicosis group.



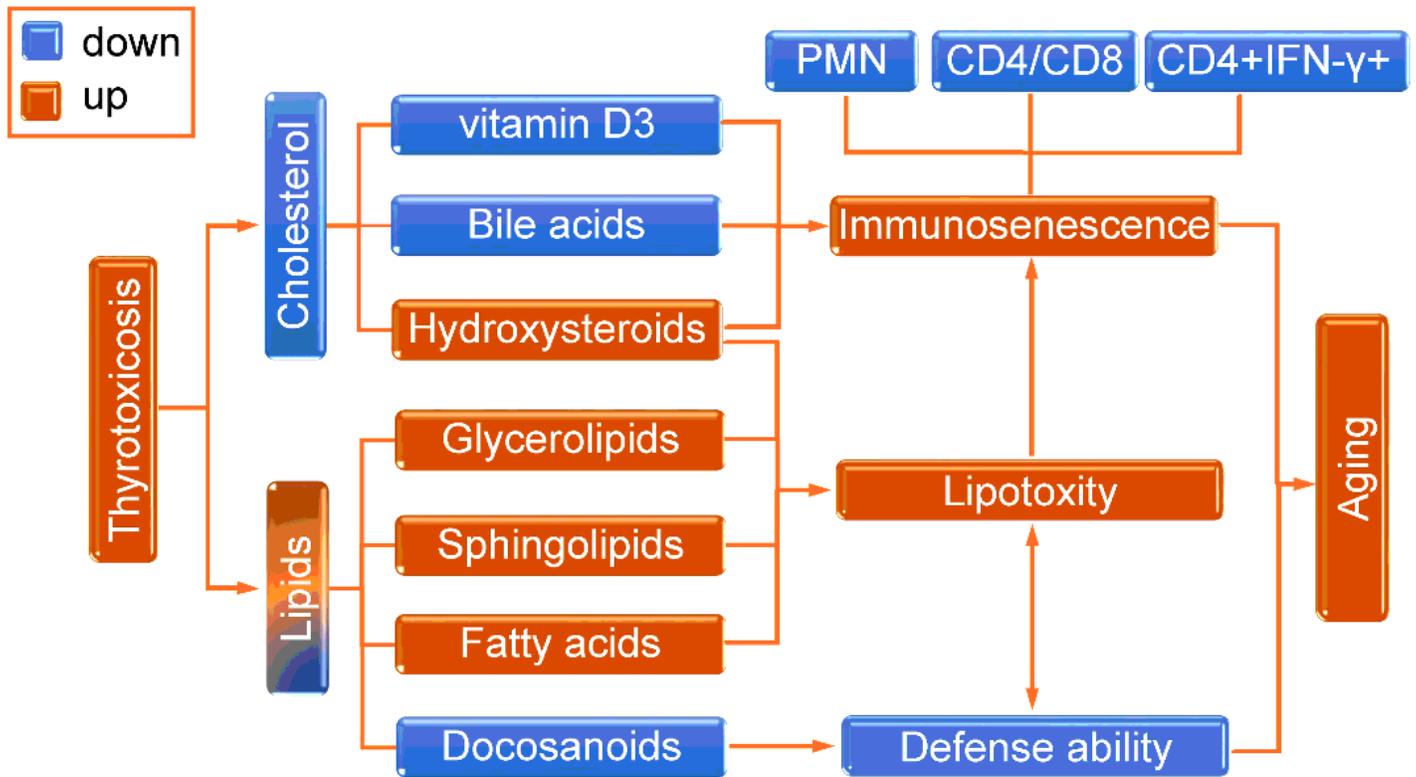
**Figure 2**

**LC-MS metabolomics analysis.** **(a)** There were significant differences in OPLS-DA score between thyrotoxicosis group (T) and normal control group (C). OPLS-DA, orthogonal partial least squares discrimination analysis. **(b)** Clustering heat map of 157 DEMs, including 81 upregulated ones and 76 downregulated ones (HMDB database). **(c)** The KEGG pathway analysis result of all DEMs. **(d)** The classification of LC-MS DEMs. **(e)** The expression of Thyroxine (T4) and lodothyronamine (T3) were significantly upregulated in thyrotoxicosis mice. **(f)** The Volcano plot of 325 lipid DEMs (Lipidmaps Database), including 240 upregulated ones and 185 downregulated ones. **(g)** The classification of LC-MS lipid DEMs (Lipidmaps Database). T: thyrotoxicosis mice; C: normal control mice.



**Figure 3**

**GC-MS metabolomics analysis.** (a) There were significant differences in OPLS-DA score between thyrotoxicosis group (T) and normal control group (C). (b) The Volcano plot of 78 lipid DEMs (HMDB database), including 13 upregulated ones and 65 downregulated ones. (c) The KEGG pathway analysis result of all DEMs. (d) The classification of GC-MS DEMs. T: thyrotoxicosis mice; C: normal control mice.



**Figure 4**

**The relationship of disordered lipid metabolism with immunosenescence in thyrotoxicosis mice** Serum metabolomics analysis showed that the metabolism of thyrotoxicosis mice was disordered, especially the synthesis and metabolism of cholesterol and lipids. The decrease in cholesterol synthesis and absorption led to a decrease in serum cholesterol, which consequently resulted in a decline in the levels of cholesterol derived VD and bile acids. The lack of VD and bile acids led to the decline of immunity. In contrast, another type of cholesterol derivative GCs increased significantly, which can cause immunosenescence. A variety of lipotoxicity related metabolites were up-regulated, including Glycerolipids, Sphingolipids and Fatty Acids. Lipotoxicity is mainly manifested in its damage to the mitochondrial membrane, acting on immune cells leading to immunosenescence. Furthermore, GCs are also Lipids and lipid-like molecules, and the excessive GCs can also be classified as lipotoxicity factors. On the contrary, protective Docosanoids were downregulated, but correspondingly, their decrease weakens their ability to resist lipotoxicity, leading to the decline of defense ability. In conclusion, present study showed that immunosenescence was found in thyrotoxicosis mice, and lipotoxicity may be one cause, which provides a basis for simulating aging in the thyrotoxicosis mice model.