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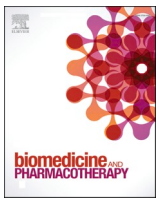

Network pharmacology and topological analysis on tibolone metabolites and their molecular mechanisms in traumatic brain injury

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ABSTRACT

Traumatic brain injury (TBI) is a pathology of great social impact, affecting millions of people worldwide. Despite the scientific advances to improve the management of TBI in recent years, we still do not have a specific treatment that controls the inflammatory process after mechanical trauma. The discovery and implementation of new treatments is a long and expensive process, making the repurpose of approved drugs for other pathologies a clinical interest. Tibolone is a drug in use for the treatment of symptoms associated with menopause and has been shown to have a broad spectrum of actions by regulating estrogen, androgen and progesterone receptors, whose activation exerts potent anti-inflammatory and antioxidant effects. In the present study, we aimed to investigate the therapeutic potential of the tibolone metabolites 3α-Hydroxytibolone, 3β-Hydroxytibolone, and Δ4-Tibolone as a possible therapy in TBI using network pharmacology and network topology analysis. Our results demonstrate that the estrogenic component mediated by the α and β metabolites can regulate synaptic transmission and cell metabolism, while the Δ metabolite may be involved in modulating the post-TBI inflammatory process. We identified several molecular targets, including *KDR*, *ESR2*, *AR*, *NR3C1*, *PPARD*, and *PPARA*, which are known to play critical roles in the pathogenesis of TBI. Tibolone metabolites were predicted to regulate the expression of key genes involved in oxidative stress, inflammation, and apoptosis. Overall, the repurposing of tibolone as a neuroprotective treatment for TBI holds promise for future clinical trials. However, further studies are needed to confirm its efficacy and safety in TBI patients.

1. Introduction

Traumatic brain injury (TBI) is one of the leading causes of death worldwide, being drug-drink-driving one of the main fatalities in Ireland. Despite being a pathology that mainly affects young people, children and people aged over 70 also have a high risk of suffering some type of accident (i.e falls) that can affect brain structures by causing a mechanical impact leading to the activation of various inflammatory and oxidative pathways during the secondary injury. Furthermore, in more chronic periods, TBI is also considered a risk factor for the development of neurodegenerative pathologies, including Alzheimer's disease and other dementias [1–4].

The main hallmark of TBI is the activation of multiple inflammatory pathways, which makes the implementation of more specific treatments particularly complex and difficult to control or mitigate the secondary damage. A high demand for energetic substrates (i.e. glucose) for post-TBI cellular repair, associated with an imbalance of calcium flux and oxidative stress after the initial trauma [5–8], leads to massive mitochondrial dysfunction resulting in cell death not only of neurons but also of astrocytes, which are cells that provide vital support to neurons in pathological conditions.

Because of the trauma affecting various cerebral structures, hormonal dysregulation is often observed in patients within the first days of the injury [9–11]. This significant reduction of hormones in the blood and the brain (hypogonadism) is particularly critical for brain remodeling, as emerging evidence suggests the vast neuroreparative effects of sex hormones [12], such as estrogens, androgens and progesterone. For instance, animals treated with estradiol can release less pro-inflammatory cytokines (i.e. TNF-a and IL-6) [13], whose increase is more pronounced in the chronic period of the lesion [7,14]. Although these hormones exert their actions by binding to their respective receptors, which are widely distributed in the brain, their therapeutic

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potential in TBI is still under-explored, especially considering their inherent risks associated with long-term use.

Tibolone is a synthetic steroid that is currently used by post-menopausal women to alleviate symptoms of osteoporosis and menopause. Once metabolized to 3α-Hydroxytibolone, 3β-Hydroxytibolone, and Δ4-Tibolone, it acts on estrogen (ER), androgen (AR), and progesterone (PGR) receptors. While the first two metabolites bind to the alpha (ERα) and beta (ERβ) estrogen receptors, the latter binds with the androgen and progesterone receptors, making tibolone a multitarget prodrug with a broad spectrum of anti-inflammatory actions that may stimulate several neurotherapeutic pathways dependent on the activation of these receptors. In previous studies by our group, we demonstrated that tibolone has protective properties in both astrocytes and neurons, reducing oxidative stress and improving mitochondrial function after metabolic damage and lipotoxicity [15–24]. Using network pharmacology we also previously showed that IL6 might be a pharmacological target of tibolone in TBI [17]. Since there is currently no cure for the sequelae and motor consequences of TBI, future therapeutic approaches must target multiple pathways to alleviate primary brain injury and reduce secondary oxidative damage. The present study builds up on a previous study from our group showing the molecular mechanisms of tibolone in TBI [17]. In here, we used an in-depth topological approach to investigate the benefits of using tibolone metabolites as a possible multitarget therapy for TBI, which in the future could be translated to clinical trials to repurpose this drug that can be administered or included in treatment plans more efficiently and quickly.

2. Materials and methods

2.1. Lists of genes regulated by tibolone metabolites and TBI-related genes

As a first approach, we used gene databases that may have some involvement in the pathology based on experimental and validated data. The CTD database (Comparative Toxigenomics Database, accessed on 28 November 2022), publicly available at www.ctdbase.org, is integrated with curated information on pathways and how they are influenced by drugs in various pathologies. To retrieve TBI-related genes, we used the keywords "traumatic brain injury" and "brain contusion" in humans (Homo Sapiens). Out of a total of 2021 genes, 1626 were selected for TBI and 395 for contusion.

Although CTD has a high reliability index, as it is based on experimental data, it is still possible to have a certain level of false positive ratio. To confirm and validate the previously obtained list, we used other databases such as Open Targets Platform (https://platform.opentargets.org), using the search terms "brain injury" and "brain concussion" we obtained 1259 and 49 genes, respectively. Finally, we used OMIM (https://www.omim.org, accessed on 28 November 2022) with 11 genes, and Malacards (1742 genes) using "traumatic brain injury" in humans as keyword.

To retrieve the molecular targets of tibolone metabolites, we first considered the SMILES (**S**implified **M**olecular **I**nput **L**ine **E**ntry **S**ystem) of each molecule obtained from PubMed (https://pubmed.ncbi.nlm.nih.gov), 3α-Hydroxytibolone (α-tibo, PubChem CID 10087021), 3β-Hydroxytibolone (β-tibo, PubChem CID 9839851), and Δ4-Tibolone (Δ-tibo, PubChem CID 22814761) and searched for related genes using Similarity Ensemble Approach (SEA, https://sea.bkslab.org/), Swissprediction (http://www.swisstargetprediction.ch/?), TargetNet (http://targetnet.scbdd.com/) and BindingDB (https://www.bindingdb.org/bind/index.jsp). For α-tibo, SEA (4 genes), SwissPrediction (100 genes), Targetnet (618 genes), and BindingDB (4 genes) were obtained, while for β-tibo, SEA (4), SwissPrediction (100), Targetnet (618), and BindingDB (4) were obtained. For the Δ-tibo metabolite, SEA (16), SwissPrediction (100), Targetnet (618), and BindingDB (10) were obtained.

2.2. Gene functional enrichment

The analysis of functional enrichment (GO – Gene Ontology) and biological processes and pathways (KEGG – Kyoto Encyclopedia of Genes and Genomes) [25] was performed using two servers. One of them, Enrichr (https://maayanlab.cloud/Enrichr/enrich) [26] is one of the classical tools for annotating gene classes to certain enriched functional terms related to biological processes, cell compartment, and molecular function. Additionally, we used ShinyGO (v.0.76, http://bioinformatics.sdstate.edu/go) and KEGG to explore those high-level GO terms and pathways in common between these web servers with the aim of validating the results found in Enrichr.

2.3. Meta-analysis of clustered biological terms

For the genes in common across all lists (TBI, α-tibo, β-tibo, and Δ-tibo), we performed a meta-analysis of enriched terms to determine the integration of the most important and critical ones using Metascape (version 3.5) [27]. This online server makes use of KEGG, Reactome [28] among others, and automatically clusters enriched terms into groups that are non-redundant, a similar approach used in DAVID [29]. The results obtained are processed based on Kappa-test score [30], where the enriched terms are computed and hierarchically classified into clusters of similar terms with a 0.3 similarity threshold and with the most significant p-value within each cluster. Finally, in this study, a functional list of clustered genes was generated to identify the most significant terms that may determine the impact of tibolone and its metabolites on those genes related to TBI.

2.4. Protein-protein interaction (PPI) network

We created a PPI network in Cytoscape (version 3.9) [31] using the final list of genes obtained from databases with the aim of identifying those genes that are essential for network stability (hubs) and building subclusters through Molecular Complex Detection (MCODE) [32] to find closely connected components in the PPI network. Initially, we used the STRING built-in plugin with a confidence cutoff of 0.4 and zero additional interactors, considering *Homo sapiens* as the organism. Being one of the most reliable meta databases that integrates validated data and predictions of gene/gene, protein/protein interactions, STRING associates the functionality of each element with its sub-localization in tissues and cell types, thus being an important resource to determine at the molecular level the impact of the network on a particular cellular compartment.

To explore other parameters of PPI that allow us to identify cellular hubs, we used Cytoscape built-in Network analysis tool, which provides topological analyses such as degree (DG), betweenness (BS), and closeness centrality. In general terms, degree refers to the number of interactions between different nodes and edges of the network, while betweenness is defined as the flow of information through a particular node in the network [33,34]. Therefore, nodes with high degree and betweenness can be considered critical hubs in the PPI. Closeness centrality (CS) refers to the distance of the flow of information between these two nodes, and the smaller this distance, the better the interaction [35]. To determine the hubs, we used MCODE in Cytoscape, which generates a subcluster using several algorithms and identifies the seed (i.e., the most critical node) and the clustered genes.

To further deepen the topological analysis of the network, we used cytoHubba [36], another plugin in Cytoscape. This tool explores the interactome and uses additional algorithms to MCODE as a tool to validate important nodes. Among these algorithms, in the present study we analyzed Maximal Clique Centrality (MCC), Maximum Neighborhood Component (MNC), clustering coefficient (CC), Density of Maximum Neighborhood Component (DMNC).

2.5. Statistical analysis

Graphpad (version 9.0) was used for statistical analysis and graphing data. Pearson's Correlation was assessed to elucidate the association between DG, BS, CS DMNC, MNC, and MCC. For all correlations, the coefficient of determination (R^2) and p-value were included.

3. Results

3.1. Enriched terms in TBI pathology

One of the most immediate consequences produced by the mechanical damage caused by trauma is persistent inflammation that begins in the acute phase and persists into the chronic phase, causing massive death not only of neurons but also of astrocytes and microglia. To better understand and investigate the molecular signature underlying TBI pathology, and how tibolone and its metabolites can regulate the genes involved in cell death caused by TBI, a Venn diagram was generated to identify those genes that are shared in at least two lists, with a total of 860 genes appearing to be related to TBI and were therefore used for further analysis. Using same approach, 120 genes for α-tibo, 121 for β-tibo, and 111 for Δ-tibo had been found shared in at least two lists (Suppl Fig. 1A–D).

We crossed the final lists of genes that actively participate in the TBI pathophysiological mechanism, and those that are experimentally validated to be modulated by α-tibo, β-tibo, and Δ-tibo (Fig. 1A and Table 1). The Venn diagram shows that 804 proteins are unique to TBI, while 2 are unique to α-tibo, 2 to β-tibo, and 32 to Δ-tibo lists.

Next, we characterized the biological processes in which these 804 unique proteins may participate in the pathology. We hierarchized the enriched terms according to the Odds ratio, which helps quantify the strength of the association between all genes and categorized GO terms. Odds ratio greater than 1 indicates a strong positive association. Our results show that out of a total of 4585 enriched terms (Fig. 1B), the top 10 include regulation of macrophage proliferation [(GO:0120040), -log10(p-value) = 5.25], response to lipoteichoic acid [(GO:0070391), -log10(p-value) = 5.25], positive regulation of interleukin-23 production [(GO:0032747), -log10(p-value) = 6.44], terpenoid metabolic process [(GO:0006721), -log10(p-value) = 6.44], positive regulation of glial cell migration [(GO:1903977), -log10(p-value) = 7.65], regulation of interleukin-23 production [(GO:0032667), -log10(p-value);= 8.86], peptidyl-cysteine S-nitrosylation [(GO:0018119), -log10(p-value) = 5.94], regulation of hippocampal neuron apoptotic process [(GO:0110089), -log10(p-value) = 5.94], positive regulation of microglial cell migration [(GO:1904141), -log10(p-value) = 7.21], and regulation of microglial cell migration [(GO:1904139), -log10(p-value) = 8.47].

To confirm and validate the previous results, we used other databases such as ShinyGO and KEGG to examine those enriched terms that describe the biological processes carried out by those 804 proteins in the TBI list. Within the enriched biological processes hierarchized according to the False Discovery Rate (FDR), response to oxygen-containing compound [-log10(FDR) = 171.54], regulation of cell death [-log10 (FDR) = 164.47], regulation of programmed cell death [-log10(FDR) = 151.59], and apoptotic processes (-log10(FDR) = 149.38) make the top regulated categories (Fig. 1C). Similar to enriched terms in Fig. 1B–C, KEGG enrichment results confirm apoptosis [-log10(FDR) = 48.41], inflammatory signaling involving TNF pathways [-log10(FDR) = 47.34], cytokine and interleukins, in particular IL17 [-log10(FDR) = 41.23], and survival MAPK-PI3K-Akt pathways [-log10(FDR) = 54.44] within the top 10 terms (Fig. 1D).

The analysis of the top 10 enriched terms (Fig. 1B) reveals the

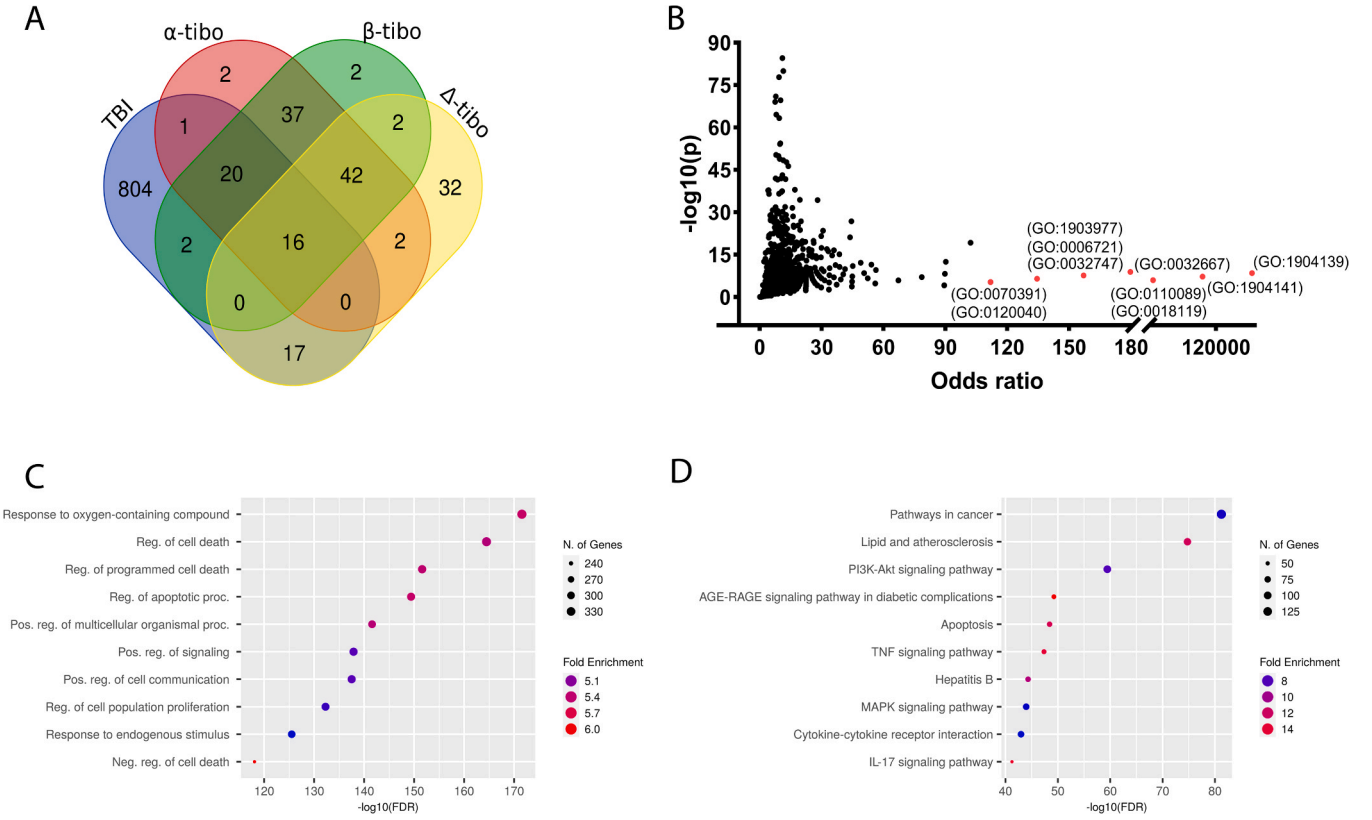

Fig. 1. Analysis of biological processes in Traumatic Brain Injury (TBI) that are molecularly regulated by tibolone metabolites. A Venn diagram is presented, depicting the genes that are present in each list, as well as the intersection between them (A). Enrichr results are also presented, showing the enriched terms, with the top 10 indicated in red according to the Odds Ratio (B). Functional enrichment using Gene Ontology (GO) (C) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (D) are shown, presenting the top enriched terms based on the number of genes and fold enrichment.

Table 1
Top biological enriched terms and genes correlated to TBI pathology.

Enriched terms	Log10	Odds ratio	Genes
regulation of macrophage proliferation (GO:0120040)	5.258905656	111.9239766	CSF1R;IL33; MAPK1;PTK2; MAPK3
response to lipoteichoic acid (GO:0070391)	5.258905656	111.9239766	LBP;CD14; MAPK14;RELA; TLR2
positive regulation of interleukin-23 production (GO:0032747)	6.441370134	134.4660422	CSF2;IFNG; CLEC7A;MYD88; IL17A;IL17RA
terpenoid metabolic process (GO:0006721)	6.441370134	134.4660422	CYP2C9;CYP2D6; CYP1A2;CYP2E1; CYP3A4;FDFT1
positive regulation of glial cell migration (GO:1903977)	7.657483307	157.0609613	P2RY12;P2RX4; CSF1;CCL3; TREM2;CX3CL1; CCR2
regulation of interleukin-23 production (GO:0032667)	8.86956964	179.7089202	CSF2;IFNG; CLEC7A;RAC1; TLR4;MYD88; IL17A;IL17RA
peptidyl-cysteine S-nitrosylation (GO:0018119)	5.949997762	95,700	NOS2;NOS1; S100A9;GAPDH; S100A8
regulation of hippocampal neuron apoptotic process (GO:0110089)	5.949997762	95,700	CX3CR1;TYROBP; ITGAM;TREM2; CX3CL1
positive regulation of microglial cell migration (GO:1904141)	7.212642176	114,840	P2RY12;P2RX4; CSF1;CCL3; TREM2;CX3CL1
regulation of microglial cell migration (GO:1904139)	8.47216498	133,980	P2RY12;CX3CR1; P2RX4;CSF1;CCL3; TREM2;CX3CL1

presence of several common proteins that are known to participate in post-TBI inflammation. These proteins include transcription factors RELA Proto-Oncogene, NF-KB Subunit (RELA), cytokines C-C Motif Chemokine Ligand 3 (CCL3), C-X3-C Motif Chemokine Ligand 1 (CX3CL1) and C-C Motif Chemokine Receptor 2 (CCR2), interleukins Interleukin 17A (IL17A) and interleukin 17 Receptor A (IL17RA), receptors Triggering Receptor Expressed On Myeloid Cells 2 (TREM2), Toll Like Receptor 4 (TLR4) and Toll Like Receptor 2 (TLR2), and cell survival pathways such as Mitogen-Activated Protein Kinase 1 (MAPK1) and 14 (MAPK14) (Table 1).

3.2. Estrogenic regulatory mechanisms of TBI pathology

Hypogonadism is a common feature in TBI patients [9–11], with some studies showing that it can be a key factor in increasing inflammation and cell death [37]. The stimulation of pathways regulated by estrogen receptors has a broad neuroprotective spectrum in various central nervous system pathologies (for full review please see [12,38,39]), including TBI [7]. In the present study, we focused on tibolone, a drug used to alleviate symptoms associated with menopause and which has been shown by our group to have extensive anti-inflammatory and antioxidant properties [15,40], as a candidate for drug repurposing in TBI. On the other hand, although tibolone protects neurons and reduces astrocyte activation by alleviating inflammation after TBI in mice [41], it is still unknown which of its metabolites, α, β, or Δ, may exert such protective effects on this pathology.

Regarding the shared genes in α-β-tibo/TBI lists, the top 10 enriched terms (Fig. 2A) include negative regulation of voltage-gated calcium channel activity [(GO:1901386), -log10(p-value) = 5.61], negative regulation of cytosolic calcium ion concentration [(GO:0051481), -log10(p-value) = 3.82], monoterpenoid metabolic process [(GO:0016098), -log10(p-value) = 3.82], response to cocaine [(GO:0042220), -log10(p-value) = 3.97], response to morphine [(GO:0043278), -log10(p-value) = 3.97], response to alkaloid [(GO:0043279), -log10(p-value) = 5.98], phospholipase C-activating dopamine receptor signaling pathway [(GO:0060158), -log10(p-value) = 5.98], prepulse inhibition [(GO:0060134), -log10(p-value) = 6.13], regulation of dopamine uptake involved in synaptic transmission [(GO:0051584), -log10(p-value) = 8.20] and regulation of catecholamine uptake involved in synaptic transmission [(GO:0051940), -log10(p-value) = 6.40].

The results of ShinyGO analysis (Fig. 2B) validate trans-synaptic signaling as top regulated enriched term (-log10(FDR) = 16.72), followed by synaptic transmission [-log10(FDR) = 16.68], chemical synaptic transmissions synaptic signaling [-log10(FDR) = 15.43], and anterograde trans-synaptic transmission [-log10(FDR) = 15.43]. Similar findings were obtained when submitting the 20 shared proteins to the KEGG database (Fig. 2C), with neuroactive ligand-receptor interaction as the top enriched term [-log10(FDR) = 18.95], followed by cAMP signaling [-log10(FDR) = 7.62], calcium signaling [-log10(FDR) = 7.52], dopaminergic synapse [-log10(FDR) = 5.66] amongst others. The comparison of the three databases suggests that the estrogenic metabolites of tibolone strongly influence the synaptic component, particularly dopaminergic signaling. DRD1, DRD2, DRD3, and DRD4 dopamine receptors are the most highly regulated proteins by these metabolites in the terms (Table 2) and are also implicated in TBI.

3.3. Androgenic and progestogenic modulation of TBI pathology

In addition to exerting estrogenic activity, Δ-tibo can activate signaling mediated by androgen and progesterone receptors [42–45]. To determine the differences in the regulation of protective pathways mediated by these receptors in TBI, we focused on examining which biological processes are regulated by Δ-tibo that can explain, at a molecular level, the broad neuroprotection mediated by these receptors [46–49]. Of the 661 biological processes regulated by Δ-tibo in TBI, the top 10 (Fig. 3A) are comprised of prostaglandin transport [(GO:0015732), -log10(p-value) = 1.70], regulation of vascular wound healing [(GO:0061043), -log10(p-value) = 3.22], negative regulation of gene silencing by miRNA [(GO:0060965), -log10(p-value) = 4.83], negative regulation of catecholamine secretion [(GO:0033604), -log10(p-value) = 3.30], regulation of norepinephrine secretion [(GO:0014061), -log10(p-value) = 3.30], positive regulation of fever generation [(GO:0031622), -log10(p-value) = 3.40], regulation of epinephrine secretion [(GO:0014060), -log10(p-value) = 3.40], receptor transactivation [(GO:0035624), -log10(p-value) = 3.50], positive regulation of heat generation [(GO:0031652), -log10(p-value) = 3.50], and regulation of fever generation [(GO:0031620), -log10(p-value) = 3.50].

To confirm the results above, ShinyGO's enrichment depicts, amongst others, inflammatory response [-log10(FDR) = 3.46] and cytokine-mediated signaling pathway [-log10(FDR) = 3.46] within the top 10 biological enriched terms (Fig. 3B). Similarly, according to KEGG results IL-17 signaling pathway [-log10(FDR) = 4.22] is the top enriched category, in addition to other similar pathways such as TNF-signaling pathway [-log10(FDR) = 2.83] (Fig. 3C), prostaglandin transport is included in the top 10 of the 661 biological processes regulated by Δ-tibo in TBI (Fig. 3A).

When analyzing which genes appear most frequently in all categories within the top 10, prostaglandin-Endoperoxide Synthase 2 (PTGS2), Tumor Necrosis Factor (TNF), Adrenoceptors Alpha 2A (ADRA2A) and C (ADRA2C) have the strongest evidence and involvement in inflammation (Table 3).

3.4. Combined effects in TBI

One of the main hypotheses is that tibolone is a multi-target prodrug with a broad effect in TBI due to the multiple regulatory effects of its

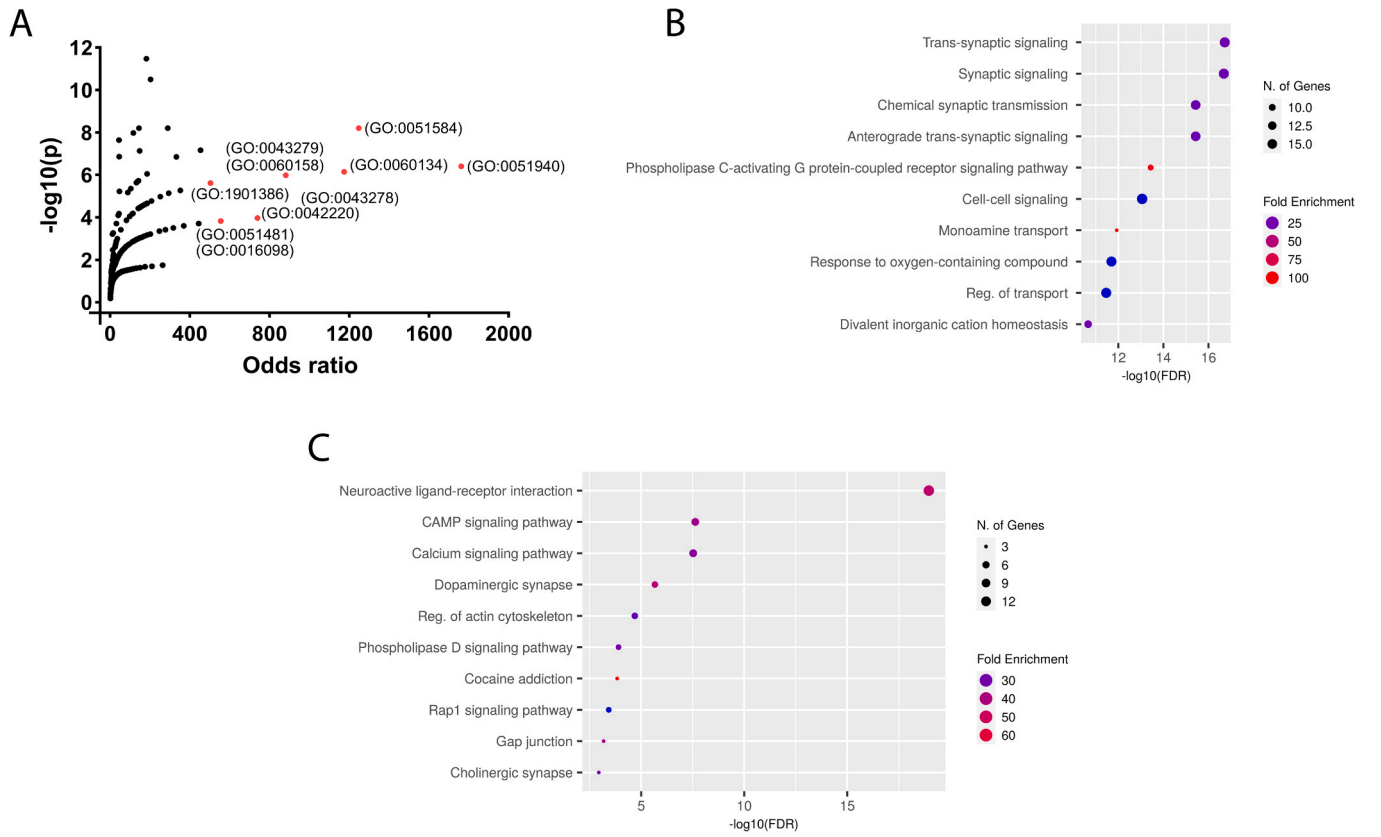

Fig. 2. Mechanisms of cellular function regulation by α-tibo and β-tibo in Traumatic Brain Injury (TBI). Enrichr database was used to determine the top 10 enriched terms according to the Odds Ratio, with the results presented in red (B). Functional enrichment using Gene Ontology (GO) (B) and KEGG (C) was performed to determine the top 10 enriched terms based on the False Discovery Ratio (FDR).

Table 2
Top biological enriched terms and genes predicted to be modulated by α- and β-tibo in TBI pathology.

Enriched terms	Log10	Odds ratio	Genes
negative regulation of voltage-gated calcium channel activity (GO:1901386)	5.61056893	503.5210084	DRD2; DRD3;DRD4
negative regulation of cytosolic calcium ion concentration (GO:0051481)	3.821296203	554.8888889	DRD2;DRD3
monoterpenoid metabolic process (GO:0016098)	3.821296203	554.8888889	CYP2C9; CYP2D6
response to cocaine (GO:0042220)	3.971321442	739.8888889	DRD2;DRD3
response to morphine (GO:0043278)	3.971321442	739.8888889	DRD2;DRD3
response to alkaloid (GO:0043279)	5.987251797	881.2941176	TRPV1; DRD2;DRD3
phospholipase C-activating dopamine receptor signaling pathway (GO:0060158)	5.987251797	881.2941176	DRD1; DRD2;DRD3
prepulse inhibition (GO:0060134)	6.139840289	1175.117647	DRD1; DRD2;DRD3
regulation of dopamine uptake involved in synaptic transmission (GO:0051584)	8.206237351	1248.5	DRD1; DRD2; DRD3;DRD4
regulation of catecholamine uptake involved in synaptic transmission (GO:0051940)	6.405836404	1762.764706	DRD1; DRD2;DRD3

active metabolites. To investigate this, we selected those genes that are shared between the α-tibo, β-tibo, and Δ-tibo lists associated with TBI. A total of 16 genes (Fig. 1A) that are modulated by estrogen, androgen and progesterone receptors and that may have a role in the pathology were found, including Peroxisome Proliferator Activated Receptor Delta (PPARD), 3-Hydroxy-3-Methylglutaryl-CoA Reductase (HMGCR), Kinase Insert Domain Receptor (KDR), Solute Carrier Family 6 Member 4 (SLC6A4), Peroxisome Proliferator Activated Receptor Alpha (PPARA), Butyrylcholinesterase (BCHE), Tachykinin Receptor 1 (TACR1), Progesterone receptor (PGR), Nuclear Receptor Subfamily 3 Group C Member 1 (NR3C1), Opioid Receptor Mu 1 (OPRM1), Janus Kinase 2 (JAK2), estrogen receptor alpha (ESR1), androgen receptor (AR), estrogen receptor beta (ESR2), and Cytochrome P450 Family 3 Subfamily A Member 4 (CYP3A4) and Family 19 Subfamily A Member 1 (CYP19A1).

The GO enrichment for biological processes indicate the top 10 enriched categories, which include positive regulation of mitochondrial depolarization [(GO:0051901), -log10(p-value) = 1.63], negative regulation of response to food [(GO:0032096), -log10(p-value) = 1.63], serotonin transport [(GO:0006837), -log10(p-value) = 1.63], serotonin uptake [(GO:0051610), -log10(p-value) = 1.63], regulation of growth hormone receptor signaling pathway [(GO:0060398), -log10(p-value) = 1.63], cellular response to testosterone stimulus [(GO:0071394), -log10(p-value) = 1.63], regulation of integrin biosynthetic process [(GO:0045113), -log10(p-value) = 1.63], vascular endothelial growth factor receptor-2 signaling pathway [(GO:0036324), -log10(p-value) = 1.63], negative regulation of cholesterol storage [(GO:0010887), -log10(p-value) = 2.97], and steroid hormone mediated signaling pathway [(GO:0043401), -log10(p-value) = 9.63] (Fig. 4A and Table 4).

Using Metascape, enriched terms across the four shared lists predominantly depict steroid signaling and metabolic processes, particularly how tibolone metabolites may regulate different cellular processes related to cell growth, apoptosis, development, and cellular metabolism (Fig. 4B). To understand the relationship between these terms, a subset of them was generated and shown in the form of a network (Fig. 4C),

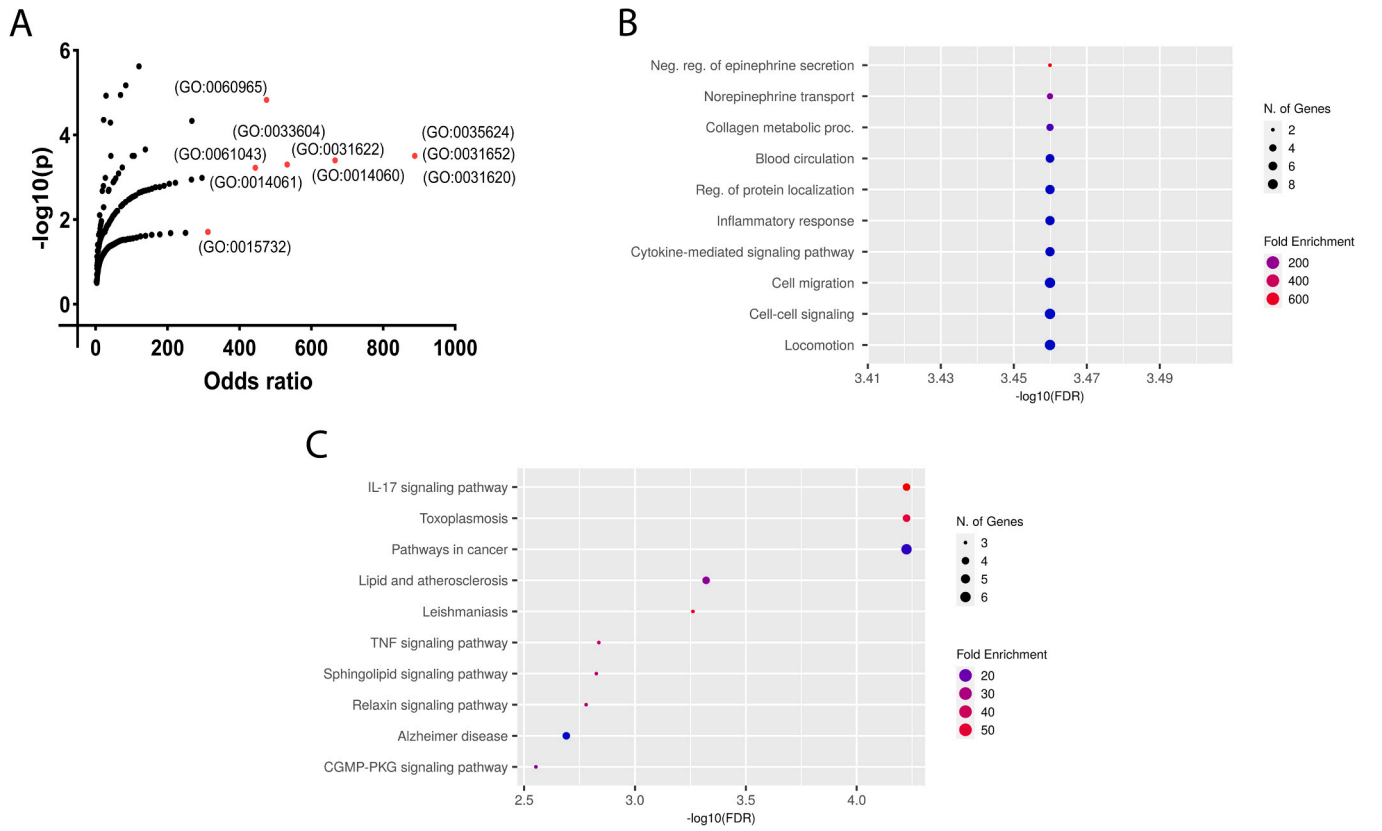

Fig. 3. Androgenic and progestogenic regulation by Δ-tibo in TBI. Characterization of the enriched biological processes performed using Enrichr (A), followed by GO (B) and KEGG (C). The red points highlighted in A represent the processes with a high Odds Ratio value.

Table 3
Top biological enriched terms and genes predicted to be modulated by Δ-tibo in TBI pathology.

Enriched terms	Log10	Odds ratio	Genes
prostaglandin transport (GO:0015732)	1.707446808	312.171875	NOS2
regulation of vascular wound healing (GO:0061043)	3.223656975	443.9333333	ALOX5;TNF
negative regulation of gene silencing by miRNA (GO:0060965)	4.830816627	475.5714286	TERT; PPARG;TNF
negative regulation of catecholamine secretion (GO:0033604)	3.304914125	532.7466667	ADRA2C; ADRA2A
regulation of norepinephrine secretion (GO:0014061)	3.304914125	532.7466667	ADRA2C; ADRA2A
positive regulation of fever generation (GO:0031622)	3.402521847	665.9666667	PTGS2;TNF
regulation of epinephrine secretion (GO:0014060)	3.402521847	665.9666667	ADRA2C; ADRA2A
receptor transactivation (GO:0035624)	3.505882556	888	ADRA2C; ADRA2A
positive regulation of heat generation (GO:0031652)	3.505882556	888	PTGS2;TNF
regulation of fever generation (GO:0031620)	3.505882556	888	PTGS2;TNF

where those with a similarity were prioritized and connected by edges. The terms with the best p-values were selected, with the restriction that there are no more than 15 terms per cluster and no more than 250 terms in general. Each node in this network represents an enriched term and is colored according to the ID of its respective cluster.

Based on the interaction network of the 16 shared proteins (Fig. 5A), MCODE analysis revealed that PPARA is the seed with a score of 7 (consisting of 7 nodes, 36 edges, and an average clustering coefficient of 0.664) and plays a critical role in maintaining homeostasis within the network (Fig. 5B and Table 5). Further analysis of the biological processes and molecular functions carried out by this subcluster revealed that DNA transcription [-log10(FDR) = 10.73] as well as steroid [-log10 (FDR) = 11.70] and lipid signaling [-log10(FDR) = 8.74] are within the top regulatory terms (Fig. 5C–D), whose cellular compartments where they occur are in the chromatin [-log10(FDR) = 7.35], in the chromosomes [-log10(FDR) = 6.52], and mitochondria [-log10(FDR) = 1.33] (Fig. 5E).

To elucidate which other transcription factors may be involved in mediating the biological processes regulated by tibolone, we utilized TRRUST, a database that identifies TF-target regulatory relationships. As shown in Fig. 5F, RELA (-log10(FDR) = 4.69) and Jun (-log10 (FDR) = 5.37) are the top two regulators in terms of the number of genes they target. PGR, PPARD, AR, and PPARA are among their main co-transcriptional targets.

Finally, using Cytohubba, we determined the number of times that the genes shown in Fig. 5A appear among the top 10 parameters such as degree, betweenness, closeness, DMNC, MCC, and MNC (Fig. 6A). These results show that KDR, ESR2, AR, NR3C1, PPARD, and PPARA are the most important genes within the network. To confirm this, we performed a correlation analysis between these different parameters. The coefficient of determination for the relationship between betweenness and degree is found to be low (R^2 = 0.3155, p-value = 0.0236) as depicted in Fig. 5B. However, a markedly higher R^2 value of 0.8161 is observed when predicting the interaction between degree and closeness (p-value < 0.0001, Fig. 5C). Similarly, Fig. 5D shows a weak correlation between degree and DMNC with an R^2 value of 0.3821, whereas a stronger correlation is observed between degree and MCC with an R^2 value of 0.7775 as depicted in Fig. 5E.

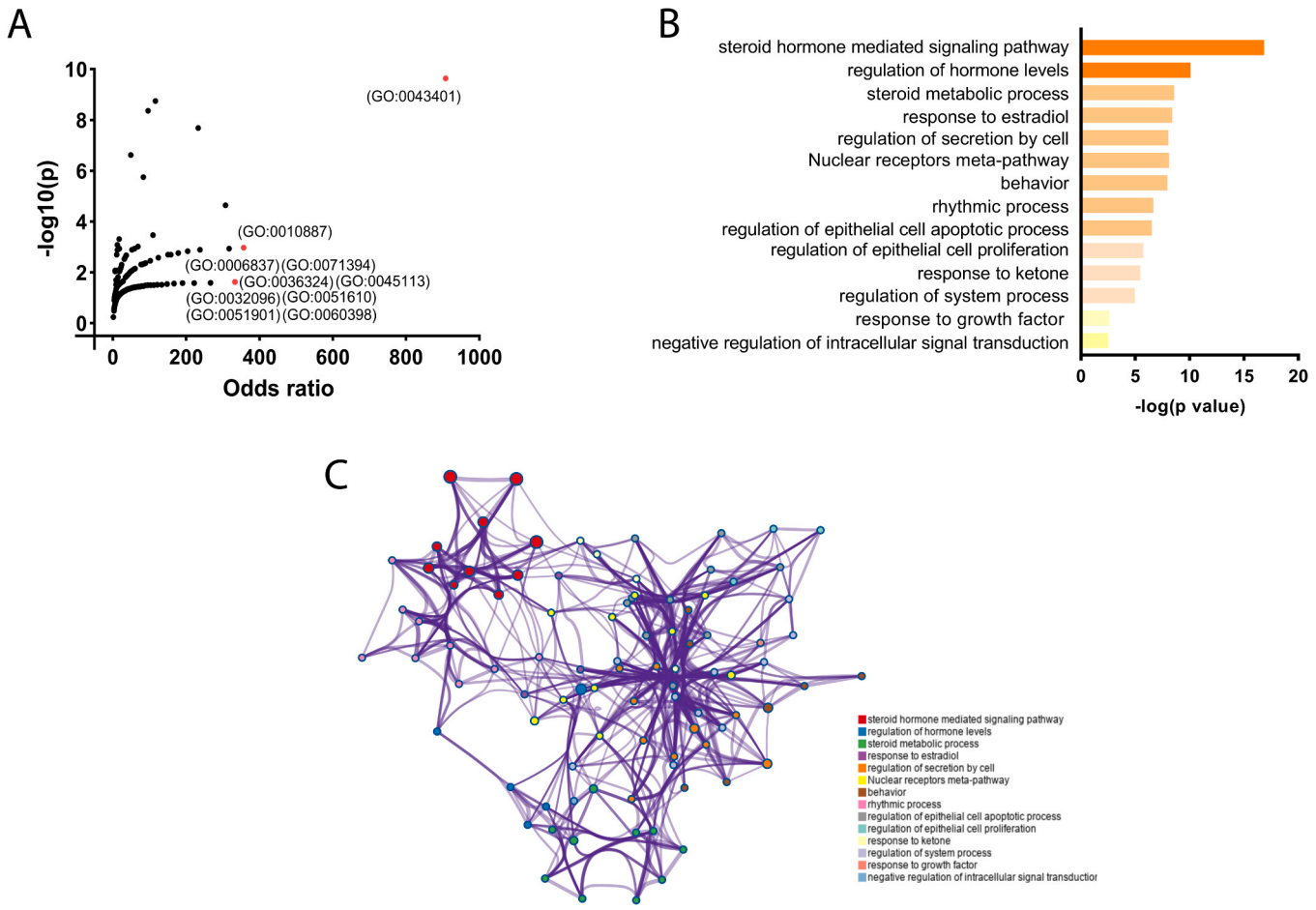

Fig. 4. Modulation of biological processes by the α, β, or Δ metabolites of tibolone in TBI. Enrichr (A) was used to explore highly enriched terms and ranked them according to the Odds Ratio, highlighting the top 10 in red. Using Metascape, the 16 genes common to all lists was submitted, and the top terms are shown in (B). To explore how these functional terms are interrelated, a PPI network was created with the aim of identifying highly connected terms that could be considered essential biological networks (C).

Table 4
Top biological enriched terms and genes shared in α-tibo, β-tibo, and Δ-tibo lists with potential involvement in TBI pathology.

Enriched terms	Log10	Odds ratio	Genes
positive regulation of mitochondrial depolarization (GO:0051901)	1.630515016	333	KDR
negative regulation of response to food (GO:0032096)	1.630515016	333	PPARA
serotonin transport (GO:0006837)	1.630515016	333	SLC6A4
serotonin uptake (GO:0051610)	1.630515016	333	SLC6A4
regulation of growth hormone receptor signaling pathway (GO:0060398)	1.630515016	333	JAK2
cellular response to testosterone stimulus (GO:0071394)	1.630515016	333	AR
regulation of integrin biosynthetic process (GO:0045113)	1.630515016	333	AR
vascular endothelial growth factor receptor-2 signaling pathway (GO:0036324)	1.630515016	333	KDR
negative regulation of cholesterol storage (GO:0010887)	2.97826772	356.7142857	PPARA; PPARD
steroid hormone mediated signaling pathway (GO:0043401)	9.638898978	907.9090909	AR;PGR; NR3C1; ESR1;ESR2

4. Discussion

The outcomes of the present study serve to complement our prior findings [17] that exclusively examined the effects of tibolone in the TBI model. The current results provide significant support to our previous findings by predicting the actions of specific tibolone metabolites in modulating various physiological processes, such as metabolism, inflammation, and synaptic mechanisms. Additionally, we conducted a more comprehensive and rigorous topological analysis of the networks, which enhances the understanding of the underlying mechanisms of tibolone.

4.1. α-tibo and β-tibo may modulate synaptic transmission while Δ-tibo inflammation post-TBI

One of the main questions of the present study was to elucidate which of the tibolone metabolites, α, β, or Δ, can regulate survival and cellular viability pathways upon TBI. Given that tibolone exerts its effects through estrogen, androgen, and progestogen receptors, we explored how each one, separately and together, can or may have an effect on inflammatory pathways modulated by TBI.

The α-tibo and β-tibo metabolites of tibolone appear to be involved in synaptic regulation and dopaminergic transmission, which play a fundamental role in inflammatory mechanisms after TBI (Fig. 2 and Table 2). It is widely known that estrogen receptors regulate several inflammation-related pathways and exert much of their protective effects on them in TBI (for a full review, please see [7,12,38]). What is not

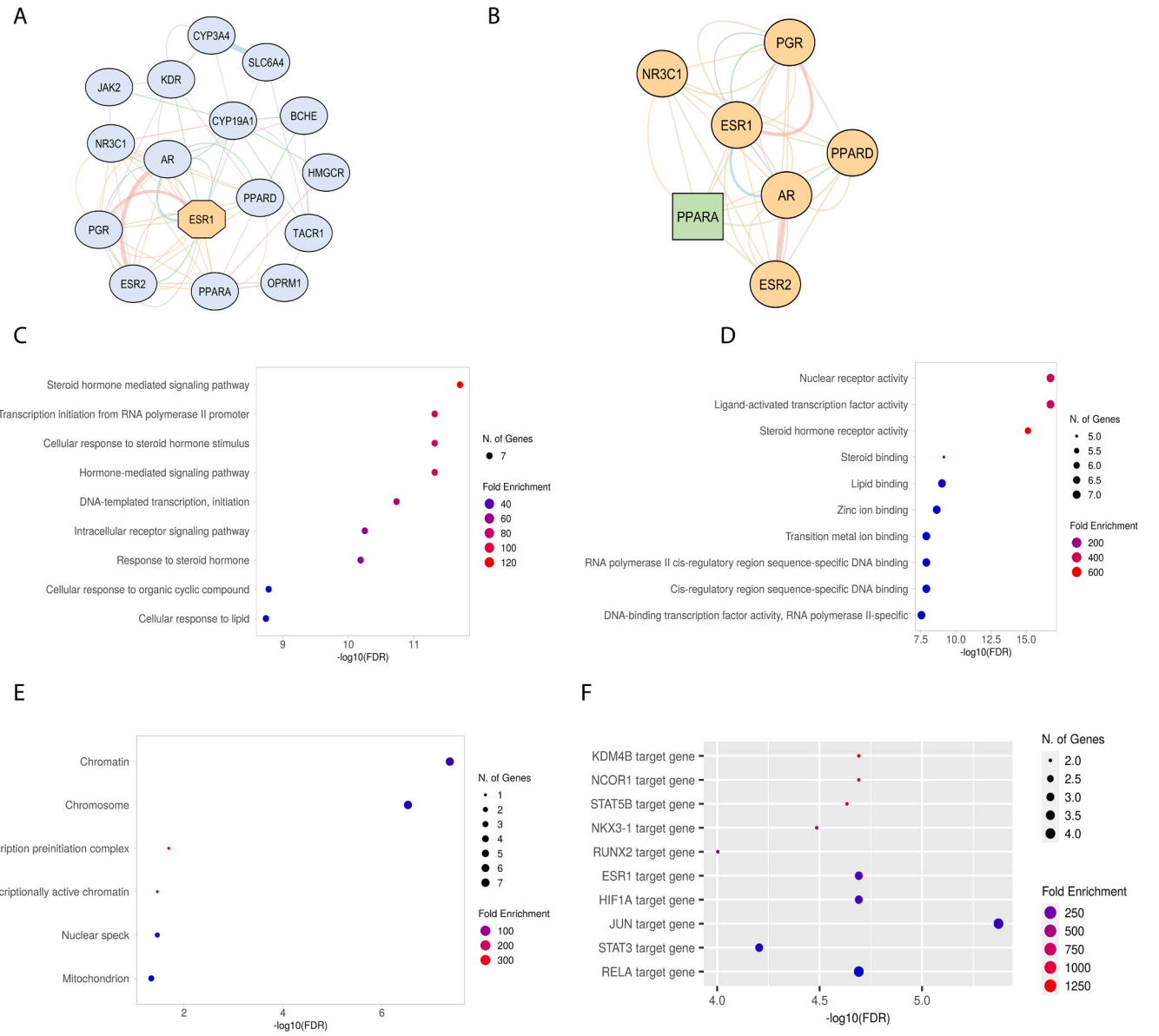

Fig. 5. Interaction network of genes in common between TBI and tibolone metabolites. A total of 16 genes were analyzed using Cytoscape to create a network, with the aim of identifying those with a high number of interactions, which can be considered biological hubs, showing ESR1 as a potential cellular node (A). MCODE analysis results show PPARA as the seed in the network (B). These proteins were then submitted to the ShinyGO server to determine the highly enriched biological processes (C), molecular function (D), and cell compartment (E) terms according to the FDR value. Lastly, the TRRUST database was used to explore other transcription factors that may be involved in regulating these biological networks (F).

yet clear is whether, similarly to the endogenous ligand, estradiol, tibolone via its estrogen metabolites may exert this same function by activating both the ERα and ERβ. As seen in Fig. 5, the cellular hubs are ESR2, ESR1, and KDR, suggesting an almost direct joint action of tibolone metabolites on both receptors and VEGF signaling (KDR), as possible neuroprotective mechanisms in TBI. Although much of the estrogenic actions of tibolone are mediated by the ERβ [12,20,21], with some studies showing an effect mediated by ERα [50], there is still no clear mechanism of tibolone on VEGF in the brain. For example, it has been reported that either tibolone or α-tibo and β-tibo metabolites have no clear effect on VEGF in breast cells [51,52], but increase signaling in adenocarcinoma cells [53], urinary tract [54], and serum [55], suggesting that this effect may be tissue- and cell-dependent.

Thinking about how tibolone may regulate mechanisms of synaptic plasticity, this area is still completely unclear, with some studies showing a positive effect of the compound [56], while others have gone further and shown that the effects of tibolone on synaptic efficacy may be dependent on the G(q) protein [57]. Finally, considering that other hubs observed in the present study relate to dopamine receptors (Table 2), it is evident that TBI greatly affects the signaling mediated by them [58], with serious consequences for synaptic transmission and plasticity during the post-TBI remodelling period. To our knowledge, there are no studies demonstrating the effectiveness of tibolone or any of its metabolites on dopamine signaling, but we can hypothesize that finding the presence of both estrogen receptors in axons and axon terminals in catecholaminergic and GABAergic neurons suggests a positive regulation of catecholamines, including dopamine [59,60]. If this can be taken into account, this same correlation could be applied to the case of tibolone, but additional studies are necessary to confirm this hypothesis.

The Δ-tibo metabolite has a joint action on androgenic and progestogenic receptors and appears to be related to alleviating the inflammatory signaling in TBI. As observed in Fig. 3 and Table 3, the inflammatory response is one of the top GO categories, and PTGS2, TNF, ADRA2A, and C are the genes with the highest evidence in enriched

Table 5
Top hubs shared in α-tibo, β-tibo, Δ-tibo lists and TBI lists. Using MCODE, PPARA is the seed within the PPI network, demonstrating a key contribution to lipid metabolism and survival pathways.

Genes	Full name	MCODE				Pathcards
		Status	Log score	Clustering coefficient	Topological coefficient	Superpathway
ESR1	Estrogen Receptor Alpha	Clustered	-0.50753749	0.5	0.468253968	Apoptosis and survival_Anti-apoptotic action, transcription Ligand-dependent activation of the ESR1/SP pathway, ERK Signaling
PPARA	Peroxisome Proliferator Activated Receptor Alpha	Seed	-0.54360745	0.571428571	0.480769231	MAPK-Erk Pathway, Nuclear receptors in lipid metabolism and toxicity, energy metabolism
NR3C1	Nuclear Receptor Subfamily 3 Group C Member 1	Clustered	-0.66735101	0.607142857	0.466666667	Circadian Clock, Immune response_IL-6 signaling pathway, Adipogenesis
PPARD	Peroxisome Proliferator Activated Receptor Delta	Clustered	-0.31564152	0.642857143	0.458333333	Fatty acid metabolism, Nuclear receptors in lipid metabolism and toxicity, Energy metabolism
AR	Androgen receptor	Clustered	-0.528913	0.761904762	0.520408163	TGF-beta Signaling Pathways, Akt Signaling, Regulation of nuclear SMAD2/3 signaling
ESR2	Estrogen Receptor Beta	Clustered	-0.39676949	0.761904762	0.510204082	Apoptosis and survival_Anti-apoptotic action of nuclear ESR1 and ESR2, ERK Signaling, MAPK-Erk Pathway
PGR	Progesterone Receptor	Clustered	-0.34429506	0.80952381	0.530612245	Signaling by ERBB4, Gene expression (Transcription), Cell cycle Regulation of G1/S transition

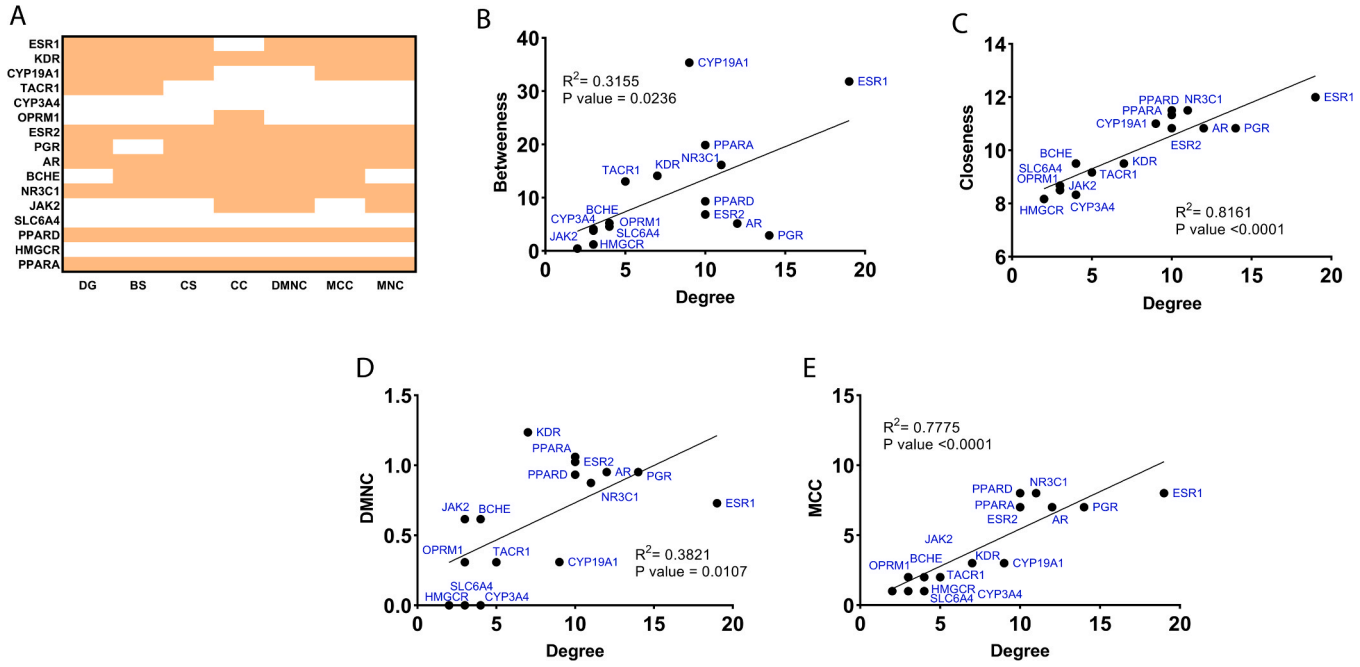

Fig. 6. Correlation between the different topological parameters of the network of 16 genes in common between TBI and tibolone metabolites. For each analyzed parameter, the number of times a gene appears within the top 10 is considered and shown in the form of a heatmap (A). Pearson's correlation was performed between degree and betweenness (B), degree and closeness (C), degree and DMNC (D), and degree and MCC (E), where the coefficient of determination and p-value are presented for each analysis.

terms. One of the studies on how androgen activation may regulate brain inflammation after a TBI came from our group when determining the actions of testosterone or its more potent metabolite, DHT, on reactive glia [47]. When administered both early (0–2 days after injury) and late (on days 5–7), both molecules reduce the number and area of immunoreactive cells for GFAP; on the other hand, only testosterone, and not DHT, is able to regulate MHC-II-labeled microglia when treatment is administered late, suggesting that the treatment window is critical to exert neuroprotection after TBI. Very similar results were observed in animals treated with 0.04 mg/Kg tibolone, where the compound also decreased the number of $GFAP^+$ astrocytes and $Iba1^+$ microglia, associated with a lower neuronal loss around the lesion 7 days after penetrating brain injury in mice [41]. Similar effects have been observed when progesterone is administered in TBI animals, and that possible effect may also be mediated by the progesterone receptor [61,62]. This androgens/progesterone protection in TBI seems to be correlated with an improvement of mitochondrial function, mainly with an increase in oxygen consumption and oxidative phosphorylation [63], which is associated with a significant decrease of hydrogen peroxide levels and increase of antioxidant proteins such as superoxide dismutase (SOD). Confirming the results in Fig. 5, previous studies from our group also show that by regulating the inflammatory pathway mediated by the NF-kB transcription factor, tibolone protects mitochondrial membrane potential, observing an improvement in cell survival and induces a positive effect on metabolic regulation in astrocytes and microglia subjected to metabolic syndrome [16,18–20,40,64,65]. In addition, the anti-inflammatory actions of Δ-tibo supports the results of our previous paper showing IL6 as a key molecular target of tibolone [17]. How the Δ-tibo influences protective mechanisms is still totally unknown, but it can be hypothesized that by promoting its effects through androgenic

and progestogenic receptors, especially considering that their activation is neuroprotective in various CNS pathologies [12,38,66,67], its precursor (tibolone) has been found to regulate IL6-dependent pathways [17,19,20] and TNF-alpha and improve antioxidant capacity in animal [50] and in vitro models [19,20].

4.2. Modulatory actions of tibolone metabolites on cell metabolism

The implication of tibolone metabolites on cell metabolism is a new finding, and for the first time we suggest that PPARD and PPARA may be its potential pharmacological targets in TBI. PPAR is a group of nuclear receptors involved mainly in the regulation of cellular metabolism [68], but has also been seen to actively participate in other processes such as dopaminergic signaling, differentiation, and cell development [69,70]. Interestingly, our results show that all three metabolites (α-tibo, β-tibo, or Δ-tibo) can exert their effects on this protein (Fig. 5), which seems to play a critical role in the post-TBI period [71–73]. PPARA agonists increase the transcription of mitochondrial enzymes, mainly those that are part of the pathway regulating fatty acid metabolism (for full review, please see [74]), besides potentiating biogenesis and mitochondrial assembly [75–77]. One may speculate that due to the presence of estrogen receptors in the mitochondria [78], and the participation of both receptors in mitochondrial metabolism and function, it is possible that there is a co-regulation of estrogen receptors and PPAR, since the former is involved in the regulation of fatty acid levels in the body [79,80]. Considering that tibolone modulates oxidative phosphorylation and metabolic processes occurring at the mitochondrial level in a sex-dependent manner in animals [15] and in cells after fatty acids lipotoxicity [16,18,64], we may hypothesize that it is possible also able to regulate PPAR-mediated pathways. However, further studies are needed on how tibolone or any of its metabolites may influence PPAR expression, and subtypes, in a specific way in each brain region and whether it is cell type dependent.

5. Conclusions

TBI is a pathology in which there is still no effective pharmacological treatment to control chronic inflammatory mechanisms, which may persist years after mechanical trauma and the onset of neurological symptoms. Although there are palliative therapeutic options to treat TBI patients, they only have specificity by having a single molecular target, whereas the pathology per se is characterized by triggering multiple oxidative mechanisms leading to a massive death of neurons and astrocytes. Therefore, it is imperative to start implementing treatments that are broader from a pharmacological point of view where they can affect several pathways that are considered critical in TBI.

In the present study, we focused on tibolone metabolites, which has been seen to have a broad spectrum of anti-inflammatory and antioxidant activity, and which can be an excellent therapeutic alternative for repurposing in TBI. We evidenced that they could regulate mitochondrial metabolism and synaptic signaling pathways and have PPARA, PPARD, and KDR as predictable molecular targets in TBI, whose effects are possibly mediated by both ERα and ERβ. With their activation, a plethora of neurochemical actions are triggered, ranging from oxidation of metabolic substrates in glycolysis and oxidative phosphorylation, mechanisms of synaptic plasticity, increase of antioxidant defense while reducing the transcription of pro-inflammatory and pro-apoptotic molecules, among others. On the other hand, we cannot rule out the participation of the androgen or progesterone receptor as part of the neuroprotective action of tibolone since they also have broad anti-inflammatory effects and may be of interest to investigate further.

The present results advance our knowledge and validate the findings and methodology employed in our previous paper by utilizing multiple topological parameters to investigate how α-tibo, β-tibo, and Δ-tibo can regulate several distinct signaling pathways that are inherently interconnected. In conclusion, tibolone metabolites possess protective properties that deserve to be further addressed as a possible therapy in TBI, due to their flexibility and specificity in stimulating biological processes mediated by ERα, ERβ, AR, and PGR whose neurotherapeutic effects actions are widely reported.

CRediT authorship contribution statement

GEB: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **JG:** Formal analysis, Writing – original draft, Resources. **DR:** Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing.

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Declaration of Competing Interest

None.

Acknowledgments

None.

Consent to publish

The authors approve the final manuscript and submission.

Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2023.115089.

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