FDA-approved drug repurposing in zebrafish identifies thyroid hormone and other compounds as potential antithrombotics

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ABSTRACT

Venous thromboembolism (VTE) is a highly prevalent medical condition with limited therapeutic options and an incomplete understanding of its acquired and inherited subtypes. The zebrafish is a model with the benefits of external development, fecundity, optical transparency, and hemostasis that demonstrates conservation with mammals. We utilized zebrafish as a phenotypic screening tool to identify novel therapeutic options for preventing VTE. A library of FDA-approved compounds was screened for suppression of acquired (elevated estrogen) and spontaneous (protein C deficiency) thrombosis. We found that thyroid hormone, receptor tyrosine kinase (RTK) inhibitors, and proton-pump inhibitors (PPIs) effectively modulated levels of thrombosis, particularly in the estrogen-induced model. These also showed a more favorable hemostatic profile than standard therapies, suggesting alternative mechanisms. Genome editing of thyroid hormone receptor proved that thyroid hormone action is on target. A retrospective electronic health record (EHR) analysis found that thyroid-hormone prescriptions in hormonal contraceptive users correlated with a higher VTE risk, potentially limiting direct repurposing but highlighting thyroid signaling as a pathway involved in estrogen-induced thrombosis. Together, these data identify several drug classes that can be tailored to specific subtypes of VTE and help elucidate distinct pathways driving thrombosis.

Key words

Venous thromboembolism, thrombosis, anticoagulant therapies, zebrafish, drug repurposing, FDA-approved compounds, estrogen-induced thrombosis, thyroid signaling, spontaneous thrombosis.

INTRODUCTION

Venous thromboembolism (VTE) includes deep vein thrombosis (DVT) and pulmonary embolism, characterized by the formation of pathologic intravascular blood clots in the venous system, leading to morbidity and mortality. VTE affects approximately 900,000 individuals/year in

53 the United States, and accounts for 60,000-100,000 deaths, imposing a heavy burden and costs
54 on healthcare systems.[1,2] The causes of VTE are varied, involving inherited factors such as
55 protein C (PC for protein, *proc* for gene) deficiency, and elevated estrogen levels from hormonal
56 treatments like combined hormonal contraceptives (CHCs) and pregnancy.[3] The PC pathway is
57 a crucial regulator of the coagulation cascade, playing a significant role in preventing intravascular
58 thrombosis and modulating inflammation.[4] The risk is heightened by factors such as hormonal
59 therapies, smoking, genetic thrombophilia, family history, and aging.[5-7] VTE is linked to numerous
60 complications, including clot progression, recurrence, post-thrombotic syndrome, and significant
61 bleeding due to anticoagulation.

62 Warfarin, direct oral anticoagulants (DOACs), and thrombolytic therapies are the primary
63 medications for the management of VTE, but they also pose a substantial risk of major bleeding,
64 and may carry up to a 9% fatality rate.[8,9] Despite recent advances in safer antithrombotic agents
65 (DOACs), these medications still parallel the risk profiles of warfarin, with a propensity for
66 bleeding.[10] DOACs accounted for nearly 40% of emergency department visits due to oral
67 anticoagulant bleeding, with hospitalization rates similar to those of warfarin.[11] Compounding this
68 issue is the limited variety of antithrombotic drugs compared to the breadth of pharmacological
69 classes available for other cardiovascular conditions such as hypertension, heart failure,
70 hyperlipidemia, and arrhythmia - each having at least 4-5 classes of medications, with between 2
71 to 10 agents per class. This disparity underscores the pressing medical need for innovative and
72 safer VTE treatments.[10,12]

73 To address this, we have leveraged the zebrafish model to investigate coagulation
74 disorders, which are characterized by rapid development from a single-cell to a fully formed larva
75 with a functional heart and circulatory system within 72 hours.[13,14] This rapid lifecycle, combined
76 with high fecundity and optical transparency of embryos, positions zebrafish as an efficient and
77 cost-effective platform for small molecule high-throughput screening.[13,15-17] This allows screening
78 of a living vascular system rather than isolated proteins or cells.[18,19] These “mini-organisms” have

been utilized in assays suited to various multiwell-plate formats, allowing for phenotypic-based screenings and collection of *in vivo* efficacy data, rapidly propagating research from preliminary screens to Phase I clinical trials.[20,21] The structural and functional homologies between zebrafish and mammalian coagulation factors facilitate the modeling of diverse VTE pathologies, encompassing venous thrombosis.[13,14,22,23] Furthermore, zebrafish larvae develop thrombosis in response to estrogen.[24]

Utilizing models of estrogen-induced (acquired) and spontaneous (genetic) thrombosis, we have identified 28 FDA-approved compounds from diverse drug families, with multiple agents modulating thrombosis across both models. This overlap indicates potential shared pharmacologic pathways across acquired and genetic VTE. Additionally, the identified candidate compounds were found to have a relatively lower hemostatic risk compared to standard anticoagulants. Comparative analysis revealed distinct clotting patterns between acquired and spontaneous thrombosis, suggesting pathological differences. Notably, standard-of-care therapies failed to achieve a complete anticoagulant effect in the estrogen-induced model. Thyroid hormone was found to have efficacy via on-target thyroid receptor signaling in both acquired and inherited thrombosis, which aligns with literature relating hyper/hypothyroidism to VTE risk.[25-29] A retrospective EHR review revealed that thyroid hormone prescriptions in hormonal contraceptive users correlates with increased VTE risk. In summary, we have used zebrafish to reveal pathological differences in thrombosis and limitations of current therapies, which could aid in developing safer and more effective therapies for VTE tailored for specific VTE subtypes, and suggesting thyroid signaling as a valuable mechanistic target.

METHODS

Fish strains and husbandry

Zebrafish were raised in accordance with animal care guidelines as approved by the University of Michigan Animal Care and Use Committee. Two transgenic zebrafish lines were

105 utilized, *Tg(fabp-fgb-egfp)* and *Tg(proc$^{-/-}$::fabp-fgb-egfp)*.[30] The former expresses enhanced green
106 fluorescent protein (eGFP) tagged fibrinogen, allowing for the visualization of thrombosis, while
107 the latter is also deficient in PC, serving as a model for spontaneous thrombosis.[31] All animal
108 procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the
109 University of Michigan (Protocol Number: PRO00010679). These experiments were conducted in
110 accordance with institutional and national ethical guidelines for animal research.
111
112 **Drug screening**
113 1,600 compounds from the FDA Approved & Pharmacopeia Drug Library
114 (MedChemExpress, Cat No: HY-L066) were screened for thrombotic effects in zebrafish models
115 of estrogen-induced (acquired) thrombosis. Embryos were treated at a concentration of 25 μM at
116 3 dpf. At 5 dpf, clotting in the caudal vein was assessed by an observer blinded to condition using
117 a semi-quantitative and reproducible grading system ranging from 0 to 3, indicating no thrombosis
118 through severe thrombosis.
119 For the acquired model, the *Tg(fabp-fgb-egfp)* line was additionally treated with mestranol
120 (prodrug of ethinylestradiol, 25 μM) at 4 dpf to induce thrombosis. Three embryos/compound were
121 tested in this model. For the initial screening, a Z' score was calculated according to the formula
122 $Z' = 1 - (\frac{3*SD\ of\ sample + 3*SD\ of\ control}{|Mean\ of\ sample - Mean\ of\ control|})$ where SD refers to the standard deviation of the
123 respective groups.[32] Molecules which passed a 50% reduction in thrombosis underwent
124 subsequent validation (**Supplemental Table 1**), including dose-response curves for the most
125 promising candidates (**Supplemental Table 2**) using 4-6 concentrations (max 100 μM) with at
126 least 12 embryos, and repeated at least twice. In the case of apixaban, we tested concentrations
127 up to 400 μM but did not explore further because this would have required raising the vehicle
128 control (DMSO) above 1%, which results in toxicity. To assess spontaneous (genetic) thrombosis,
129 18-24 embryos per compound were tested without mestranol, and with repetition at least twice.

130 Embryos were monitored daily for lethality and morphological abnormalities (heartbeat, blood
131 flow, pericardial edema, body curvature, and developmental delay). Any concentration that
132 induced toxicity was omitted from analysis.

133 Dose-response curves were generated by initially transforming the concentration values
134 to a log10 scale, with zero concentrations adjusted to 0.001 μM. Baseline correction was
135 performed using the response of DMSO-treated groups as controls. The regression lines for the
136 genetic thrombosis model were obtained using four-parameter log(agonist) versus response
137 models. In the acquired model, data normalization was conducted between zero and three,
138 referencing the average clotting scores of the DMSO and 25 μM mestranol-treated groups,
139 respectively, following baseline correction. From the resulting dose-response curves, the
140 inhibitory concentration values corresponding to 30% (IC_{30}) and 50% (IC_{50}) changes in thrombosis
141 grades were extrapolated. In a parallel study, corrected total cell fluorescence (CTCF) values
142 were obtained as described to compare the semi-quantitative grading system versus
143 quantification of the fluorescence signals.[33] All statistical analyses were performed using
144 GraphPad Prism software (version 9.1.0).

145

146 **Laser-mediated endothelial injury**

147 Laser-mediated endothelial injury was conducted using an Olympus IX73 inverted
148 microscope equipped with the Micropoint focusing system (Andor Technology, Belfast, UK) and
149 a pulsed dye laser, as previously described.[34] Embryos were anesthetized with 0.16 mg/mL
150 tricaine methanesulfonate at 5 dpf and then immobilized in 0.8% low-melting-point agarose on a
151 glass coverslip. Targeted ablation of the endothelium was carried out in the posterior cardinal vein
152 (PCV) at the fifth somite distal to the anal pore by delivering 99 laser pulses with a power level
153 set to 18 and observed for thrombotic occlusion up to two minutes. In parallel with the endothelial
154 injury procedure, the level of fluorescent thrombosis from each embryo was also recorded to

155 confirm that the applied concentrations of the respective drugs were within a range that mitigates
156 thrombosis.
157
158 **Knockdown studies using CRISPR/Cas9 gene editing**
159 CRISPR single-guide RNA (sgRNA) sequences targeting early exons were designed with
160 the Knockout Guide Design Tool (synthego.com). These sgRNA sequences, complexed with the
161 spCas9 enzyme, were injected into embryos at the 1-cell stage. The success of the injections was
162 verified by PCR and agarose gel electrophoresis. Sequences of the sgRNAs and PCR primers
163 are provided in **Supplemental Table 3**.
164
165 **Electronic health record (EHR) data analyses**
166 This study was approved by the University of Michigan Institutional Review Board. Using
167 the DataDirect EHR cohort discovery and data extraction software at the University of Michigan,
168 a search was conducted for female patients who had an outpatient encounter (clinic appointments
169 or emergency room visits) from January 1, 2000 to December 31st, 2019, and were between ages
170 12-50 at the time of the encounter. Patient data including smoking history, encounter dates, body
171 mass index (BMI), ICD9 (International Classification of Diseases) and ICD10 diagnosis codes
172 (billing and/or physician-entered data), outpatient medications prescribed, and comorbidities
173 which were represented via the Charlson Comorbidity Index.
174 The patients were placed in three separate categories based on which medications they
175 were prescribed during the timeframe of interest. These categories included: (1) both hormonal
176 contraception (HC) and thyroid medication (desiccated thyroid extract, levothyroxine, or
177 liothyronine), (2) HC without thyroid medications, or (3) thyroid medications without HC
178 medications. This determination was based on prescription data and included HC written date
179 (StartHC), HC end date (EndHC), along with thyroid medication written date (StartThy) and thyroid
180 medication end date (EndThy). Because liothyronine and desiccated thyroid extract have different

181 doses than levothyroxine, they were converted to levothyroxine equivalent doses.[35] Intrauterine
182 contraceptive devices and progesterone only HC were excluded. Overlap between hormonal
183 contraception and thyroid medication prescriptions was determined by using SQL code (code
184 logic available upon request).

185 After patients were placed in these different categories, the rate of thrombosis was
186 determined using thrombosis-related ICD9 and 10 events. Any diagnosis that was from a
187 historical source was excluded. Because DataDirect software includes related diagnoses (e.g.
188 chronic and acute DVT), any non-acute thrombotic event was excluded. If there were multiple
189 encounters with varying dates, the initial encounter where a thrombotic event occurred was used.
190 If no BMI or comorbidities were noted during the encounter, the nearest record was used. If there
191 were no BMI or comorbidity data documented for that patient, the field was left blank. For
192 comparative purposes, diagnoses were simplified to either DVT, pulmonary embolism,
193 thrombophlebitis, stroke, abdominal thrombosis, or arterial thrombosis. For example, DVT of the
194 left lower extremity was simplified to DVT). Full diagnoses codes and equivalent simplified
195 diagnosis key available upon request.

196

197 **Image analyses comparing differences in clotting patterns**

198 To examine clotting pattern differences between thrombosis models, we developed
199 convolutional neural network (CNN) and support vector machine (SVM) algorithms using Python
200 in a Google Colab environment. In total, 2,072 images from six independent experiments were
201 analyzed, consisting of 1,066 images from the acquired thrombosis model and 1,006 images from
202 the spontaneous model (three experiments per model).

203 For the CNN analysis, built with PyTorch, images were resized to 224 × 224 pixels. Images
204 containing minimal green fluorescence were excluded by computing the percentage of bright
205 green pixels, which reduced the dataset to 810 images. These images were later processed
206 through three convolutional layers and two fully connected layers. The data were then split into

207 80% training and 20% testing sets. This experiment was run three times, and the performance
208 metrics were averaged across runs.
209 In the case of SVM algorithm, built with scikit-learn, all images (without the green-pixel
210 filtering step) were resized to 15 × 15 pixels, flattened into feature vectors, and split into training
211 and testing sets.
212 Both models were evaluated on accuracy, balanced accuracy, area under the curve
213 (AUC), sensitivity, and specificity. The analytic codes, along with an example use case
214 walkthrough for running the models on a subset of the dataset, are available in the GitHub
215 repository (https://github.com/ymnmurat/Zebrafish-Imaging-Classification).
216
217 **Compound structure-based analysis**
218 The lead compounds identified from the high throughput screen were sorted and binned
219 based on common structural features and functional groups. Additional compounds were
220 identified by substructure searching using the Chemical Abstracts Services (CAS) SciFinder
221 Discovery Platform™ based on structural and functional group similarity.
222
223 **Statistical analyses**
224 Ordinal scale statistics were employed to test the effects of the respective treatments
225 based on observed thrombosis grades. The *MASS* library in R (v4.3.3) was utilized to derive
226 proportional odds logistic regression (polr) models as previously described.[36,37] Time to occlusion
227 analyses were conducted using multivariate Cox proportional hazards regression models with the
228 *survival* library in R.[38,39] Following the development of the respective models, marginal means
229 were estimated, and Dunnett's multiple test corrections were applied for multiple comparisons
230 using the *emmeans* library.[40]
231
232

233 **RESULTS**

234 **Distinct drug classes mitigate estrogen-induced thrombosis**

235 We employed a comprehensive library of 1,600 FDA-approved compounds and
236 pharmacopeia drugs to perform a screen for prevention of thrombosis induced by mestranol, a
237 synthetic estrogen analog used for hormonal contraception (**Figure 1A-B**). The calculated *Z'*
238 score was 0.4273 (**Figure 1C**), indicating reliable assay performance. In the first pass, a total of
239 117 compounds showed potency in reducing acquired thrombosis beyond an arbitrarily selected
240 threshold of 50% reduction, according to our grading system. These candidates underwent further
241 validation to confirm their therapeutic potential.

242 Subsequent analysis involved establishing dose-response relationships and determining
243 potencies via IC_{30} and IC_{50} measurements, while concentrations demonstrating toxicity were
244 omitted. As curves were generated using an ordinal scale (grades 0 to 3), we compared this
245 method to a fully quantitative approach by calculating Corrected Total Cell Fluorescence (CTCF)
246 values from the same images used for grading (**Figure 1D**). In a representative evaluation with
247 elbasvir and L-thyroxine treatments, we observed a high correlation (Spearman's ρ=0.85)
248 between grading and CTCF values, which supports the former system as a practical alternative.
249 However, an earlier dose response shift by the CTCF curve, in comparison to the ordinal scale
250 shift, indicated that the fully quantitative system was more sensitive to wide changes in
251 fluorescence intensity than the grading system (particularly the slope). For instance, a shift in
252 fluorescence levels caused by 5 μM elbasvir were graded as '2,' while CTCF measurements
253 indicated that there was actually more than half reduction in thrombosis. Similarly, 0.06 μM L-
254 thyroxine caused ~40% decrease in thrombosis which only became detectable with the grading
255 system at 0.13 μM.

256 Among the validated compounds, we observed distinct therapeutic classes;
257 steroid/hormone related, RTK/VEGFR inhibitors, proton-pump inhibitors, neuromodulators, and
258 immune system modulators (**Table 1, Supplemental Table 4**), demonstrating multiple

259 mechanisms available for prevention of estrogen-induced thrombosis. Interestingly, some of the
260 drugs such as esomeprazole and the RTK/VEGFR inhibitors posed pro-coagulant effects in the
261 absence of mestranol, suggesting a competition between the molecular mechanisms is induced
262 by these compounds and estrogens. Among the candidates, only thyroid hormones were able to
263 reduce thrombosis at nanomolar concentrations.
264
265 **Drug-response profiles between the acquired and genetic models overlap**
266 Parallel screening of ~800 compounds conducted in the *proc* knockout line (genetic
267 model) identified some hits found in the acquired model. Several categories of compounds,
268 including hormone-related treatments, neuromodulators, proton-pump inhibitors, and RTK
269 inhibitors, were effective across both, while others were model-specific (**Table 1, Supplemental**
270 **Table 4**). Although the two models represent distinct thrombotic processes, this overlap indicates
271 shared pharmacologic pathways that can be targeted in both acquired and genetic VTE.
272 Hormone-related compounds, such as the aromatase inhibitor exemestane and thyroid
273 hormones, demonstrated anticoagulant effects in the *proc* mutant line, while the thyroid receptor
274 antagonist (compound 1-850) showed only mild activity in estrogen-induced thrombosis and no
275 clear effect for spontaneous thrombosis. The progestin inhibitor mifepristone was notable for its
276 procoagulant profile in the genetic model as well. Neuromodulators like meclofenoxate and
277 lofepramine had a noticeable impact on acquired thrombosis but were less active in the *proc*
278 mutant. However, a cannabinoid receptor agonist AM-251 and a GABA receptor modulator
279 etomidate were effective in both.[41,42] Loxapine (dopamine and serotonin receptor antagonist), in
280 contrast, led to procoagulant effects in the absence of estrogens.[43] Elbasvir, an antiviral drug,
281 also revealed a strong anticoagulant profile for estrogen-induced thrombosis with no equivalent
282 effect in the genetic model.
283 PPIs exhibited divergent effects, with dexlansoprazole demonstrating a mild anticoagulant
284 shift relative to control in the genetic model, unlike esomeprazole, which was procoagulant.

285 SCH28080 (a non-standard PPI that structurally deviates from the archetype) was shown to
286 reduce acquired thrombosis. Overall, these data suggest a thrombosis-modulating role for proton
287 pumps. Similar to what we observed with esomeprazole, the RTK inhibitors nintedanib and
288 tivozanib displayed promising profiles in acquired thrombosis, yet procoagulant activity in *proc*
289 mutant, without any clear changes in the wild-type controls.

290 Interestingly, the immunomodulatory drugs buparvaquone, edaravone, and malotilate,
291 along with the antibiotic agents thiabendazole and nalidixic acid, demonstrated antithrombotic
292 effects in the genetic model, but were ineffective in preventing acquired thrombosis.

293 Given that standard-of-care anticoagulants, including warfarin and the DOACs, are
294 commonly prescribed for a wide spectrum of VTE cases, we next evaluated them in both models
295 and compared their effects to the candidate hits (**Table 1**). Surprisingly, there were minimal effects
296 on acquired thrombosis, which is concordant with our previous studies.[24,44] In contrast, warfarin
297 (IC_{50}: 50 nM) and dabigatran etexilate (IC_{50}: 11.72 μM) exhibited robust anticoagulant effects in
298 the *proc* mutants, consistent with their known inhibitory effects on clotting factors. Apixaban
299 reduced thrombosis by ~20%, but only at concentrations exceeding 200 μM. These outcomes
300 present an intricate drug response landscape, with certain agents showing distinct effects that
301 may align with different pathological thrombosis subtypes.

302 Upon closer examination of the control embryo images from both models, distinct clotting
303 patterns were observed (**Figure 2A**). Estrogen exposure resulted in a speckled distribution of
304 clots. In contrast, images from *proc* mutants exhibited a sprouting pattern with occasional areas
305 showing dense accumulation. These model-specific drug responses may explain why standard
306 anticoagulants fail to prevent estrogen-induced thrombosis suggesting the presence of different
307 pathological VTE subtypes. Using CNN and SVM algorithms, we were also able to distinguish
308 between the two types of thrombi with high sensitivity and accuracy (**Figure 2B**). The relatively
309 low specificity of SVM (0.69) was expected, as this approach lacked a preliminary filter to exclude

310 images with minimal fluorescence hence its deployment on all 2,072 images, including 1,125
311 graded 0-1 (none to low thrombosis).
312
313 **Candidate compounds exhibit minimal effects on hemostasis compared with standard**
314 **anticoagulants**
315 To further assess hemostatic balance and potential therapeutic efficacy, we applied a
316 laser-mediated endothelial injury assay for the candidate drugs while simultaneously confirming
317 effectiveness on thrombosis (**Figure 3**, **Supplemental Figure 1**) in comparison to the standard
318 anticoagulants. Time to occlusion (TTO) values for the candidate compounds closely aligned with
319 those of the control. Notably, significant delays in occlusion were observed for the standard
320 anticoagulants relative to negative control (as previously shown), despite no clear mitigation of
321 the acquired thrombosis phenotype at the concentrations tested.[22,45] Interestingly, some
322 candidates, including liothyronine and RTK inhibitors, delayed the occlusion times when estrogen
323 levels are elevated, yet this effect remained less pronounced than that observed with standard
324 anticoagulants, both with and without estrogens. These findings suggest that the candidate drugs
325 may effectively modulate clot formation in the acquired model with a reduced risk of bleeding with
326 respect to the approved standard anticoagulants that carry this risk inherently.
327
328 **Limited efficacy of the standard anticoagulants is likely not due to drug-drug interactions**
329 The observations of limited efficacy profiles against estrogen-induced thrombosis
330 associated with the standard-of-care therapies (apixaban, dabigatran and warfarin) raised the
331 possibility of potential drug-drug interactions with estrogens or issues with absorption. To address
332 this, simultaneous assessments of thrombosis levels and time to occlusion were performed
333 (**Figure 3**, **Supplemental Figure 1)**. Notably, treatments involving the standard anticoagulants,
334 with and without estrogens, resulted in significant delays in occlusion, suggesting effective
335 absorption of the standard anticoagulants without reducing thrombosis (**Supplemental Table 5,**

336 **Supplemental Table 6**). More striking were the estrogen/standard anticoagulant combination
337 groups, which exhibited even more pronounced delays in occlusion time. This outcome hinted at
338 a possible dynamic interaction between the clot-promoting effects of estrogens and the clot-
339 preventing actions of standard anticoagulants. The absence of delays in occlusion times in
340 estrogen-only controls further indicated that the mechanisms at play may be more complex than
341 initially assumed and warrant additional investigation.
342
343 **Candidate drugs form distinct molecular clusters based on their structures**
344 Based on the obtained compound classes, we further investigated structure-based
345 similarities between the candidates. Close similarities can be indicative of overlapping
346 mechanisms of actions of the compounds, and the relevant structures can also serve as a scaffold
347 for novel drug development strategies in the future. Accordingly, we observed distinct clusters
348 formed by thyroid hormones, RTK/VEGFR inhibitors and PPIs. Interestingly, laquinimod, an
349 immunomodulatory drug, also fell into a cluster with the RTK/VEGFR inhibitors, and the
350 arylhydrocarbon receptor (AhR) inhibitor benvitimod had structural similarities with the thyroid
351 hormones. These results suggest overlapping mechanisms with the compounds and their
352 respective clusters.
353
354 **Terminal phenyl ring iodination is required for antithrombotic activity**
355 Thyroid medications demonstrated potent antithrombotic effects and relatively low
356 hemostatic risk profiles. We therefore tested additional compounds with core structures similar to
357 thyroid hormone in both the acquired and genetic thrombosis models (**Table 2**). L-tyrosine, as
358 well as iodine addition at the 3 and 5 positions of the phenol ring (3,5-diiodo-L-tyrosine dihydrate),
359 did not affect thrombosis. Interestingly, L-thyronine, a non-iodinated analog of liothyronine, did
360 not alter thrombosis in either model. Conversely, tiratricol, which retains iodine in the terminal
361 phenyl ring but lacks the amine on the carboxyl chain, effectively modulated estrogen-induced

362 and spontaneous thrombosis with nanomolar potency (<100 nM). These observations indicate
363 that iodine groups at the terminal phenyl rings, functionally important residues in thyroid hormone,
364 play a major role in the ability of thyroid hormone to modulate thrombotic events.
365

366 **Antithrombotic effects of thyroid hormone are on-target through known receptors**

367 The iodine requirement suggested that thyroid hormone was likely mediating
368 antithrombotic effects through a thyroid hormone receptor (THR). Since medications can have
369 off-target effects, we investigated whether the thyroid hormone antithrombotic actions were
370 mediated via canonical THRs. We used CRISPR/Cas9 to knock down all known thyroid receptor
371 orthologs (*thr*) (**Figure 4**). In the controls (DMSO) and thyroid hormone (L-thyroxine) alone, there
372 were no alterations in thrombosis. However, the ability of L-thyroxine to reverse estrogen-induced
373 thrombosis was lost in the knockdown. Similarly, tiratricol was not able to prevent the estrogen-
374 induced phenotype under *thr* knockdown, strongly implicating thyroid receptor signaling
375 (**Supplemental Figure 2**). This pattern was also observed in the genetic VTE model, where L-
376 thyroxine significantly lost its antithrombotic efficacy following receptor knockdown, reinforcing
377 that these effects are receptor-mediated (**Supplemental Figure 3**).

378 We next explored whether the increased TTO observed with thyroid hormones was
379 mediated through its receptor (**Figure 5**; **Supplemental Figure 4**). Upon receptor knockdown,
380 the TTO values closely approximated baseline, suggesting that the effect is on target.
381 Interestingly, a marginal delay in TTO levels was still observed in knockdown groups that were
382 treated with L-thyroxine in combination with elevated estrogen levels or protein C deficiency,
383 indicating residual receptor activity after the *thr* knockdown.
384

385 ***thrab* is the major paralog mediating antithrombotic activity of thyroid hormone**

386 We next tested each *thr* gene individually to determine which one(s) mediate the
387 antithrombotic effect. We found that L-thyroxine treatment in *thraa* and *thrb* knockdown groups

388 could still rescue acquired thrombosis similarly to the wild-type controls (**Figure 6**). However, L-
389 thyroxine treatments failed to reduce the increased thrombosis levels in the *thrab* knockdown and
390 the triple thyroid receptor knockdown groups, indicating that *thrab* mediates the antithrombotic
391 effect.
392
393 **Electronic health records indicate clinical relevance of thyroid levels in estrogen-induced**
394 **thrombosis**
395 To determine whether our findings were clinically relevant, we undertook an analysis of
396 University of Michigan EHR data for 47,710 subjects on hormonal contraception (HC) without
397 concurrent thyroid medications (**Table 3**). 321 (0.67%) developed thrombosis in comparison to
398 27 of 2,101 (1.27%) who were concurrently using HC and thyroid medication. This comparison
399 yielded an odds ratio of 1.9 for thyroid hormone medication plus HC compared to HC alone, with
400 a number needed to harm (NNH) of 167. Additionally, 30,921 patients were taking thyroid
401 medication without HC, of which 260 (0.83%) developed thrombosis. Comparing this group to
402 those taking both HC and thyroid medication resulted in an odds ratio of 1.5 for thrombosis and
403 an NNH of 227.
404 Notably, there were some differences in the patient characteristics (**Supplemental Table**
405 **7**). For example, the patients in the thyroid medication only group were older and had higher BMIs.
406 Smoking status was similar between the HC alone group and the HC with thyroid medication
407 group, but there were more patients with unknown smoking status in the thyroid medication alone
408 group. Interestingly, the estrogen dose was higher in the HC with thyroid medication group
409 compared to the HC alone group, while the levothyroxine equivalent dose was lower in the HC
410 with thyroid medication group compared to the thyroid medication alone group.
411 Comorbidities were more prevalent in the HC with thyroid medication group compared to
412 either HC or thyroid medication alone. Chronic pulmonary disease, malignancy, and diabetes
413 were observed more in the patients who use HC along with thyroid medications. Rates of liver

414 diseases and metastatic solid tumors were also high across patients using thyroid medications
415 irrespective of HC status (**Supplemental Figure 5**). As malignancy is a well-known prothrombotic
416 state, a subanalysis was performed wherein patients with recorded cancer (labeled malignancy
417 or solid tumor) were excluded. Ultimately, this subanalysis did not reveal a significant difference
418 in odds ratio compared to the original (data not shown).

419 As observations further supported the clinical relevance of thyroid hormones in thrombosis
420 risk, it is also important to note some discrepancies. Our experimental data suggest that thyroid
421 medications may be preventive for thrombosis. However, EHR data from 27 individuals who were
422 recorded as using oral contraceptives during thyroid therapies showed higher thrombosis rates.
423 This was in comparison to those who reported using either oral contraceptives or thyroid therapies
424 alone within the same timeframe.

425

426 **DISCUSSION**

427 VTE is a common medical condition with limited treatment options, many of which come
428 with a high risk of significant bleeding. Moreover, the thrombotic effect of estrogen therapy has
429 been recognized for many years, yet we still know little about the underlying mechanisms and
430 how well its pathology aligns with conventional thrombosis. To address the need for novel
431 antithrombotics, we utilized a robust zebrafish thrombosis reporter line, enabling efficient
432 screening of 1,600 small molecules for prevention of estrogen-induced thrombosis. The semi-
433 quantitative grading system by blinded observers closely approximated quantitative fluorescence
434 readings, offering a practical solution for rapidly and reliably identifying promising drug candidates
435 and determining meaningful IC_{30} and IC_{50} values. While effective, automating the fluorescence
436 quantitation process from embryo images could significantly augment our capabilities. Such
437 automation strategies could include removing autofluorescence, accurately capturing signals from
438 each individual embryo, and enhancing signal precision. These advancements are warranted for

439 further studies to better detect subtle changes in thrombosis levels at higher grades and fully
440 exploit the throughput potential of zebrafish thrombosis models.

441 Using IC_{30} values, we captured drugs capable of marginally shifting thrombosis grades,
442 helping us identify a broad range of potential drug families for future antithrombotic development.
443 In addition, IC_{50} values helped prioritize the small molecules with the most significant effects on
444 thrombosis and identified distinct mitigating drug classes. Intriguingly, standard-of-care
445 anticoagulants failed to prevent estrogen-induced thrombosis in the regimen used in this study,
446 despite our previous findings that they completely block induced thrombosis secondary to
447 endothelial injury.[22,45] This suggests that the impact of estrogen-induced thrombosis may be
448 partially regulated by other mechanisms besides the traditional coagulation pathway. In contrast,
449 we have observed that dosing regimen and timing are important and warfarin can partially prevent
450 thrombosis when it is initiated at least two days prior to the estrogen.[24] This is likely due to the
451 known delayed onset of warfarin's effect given the differential half-lives of the vitamin K dependent
452 coagulation factors.[46] While differences in study design limit direct comparisons, these results
453 suggest that the duration of estrogen exposure and the timing of anticoagulant initiation are
454 important determinants of efficacy. Nevertheless, in both studies standard-of-care anticoagulant
455 efficacy was markedly reduced or incomplete against the extended estrogen exposure regimen,
456 whereas several of the candidate compounds we identified maintained strong antithrombotic
457 activity under the same conditions. This contrast suggests that the drug classes observed in this
458 study may offer more effective therapeutic strategies for estrogen-induced thrombosis. Notably,
459 among these drug classes, hormone-related compounds, especially thyroid hormones, showed
460 strong antithrombotic effects at very low concentrations.

461 There were a number of hits that had overlapping effects on inherited and acquired
462 thrombosis, while others affected only one system (**Table 1**, **Figure 2**). Hormone-related
463 compounds such as exemestane and thyroid hormones demonstrated antithrombotic effects in
464 both models, indicating potential mechanistic overlap. The candidate hits that were effective in

465 both models (e.g., hormone-related compounds, some neuromodulators, and PPIs) suggest that
466 their effects in estrogen-induced thrombosis are not strictly due to a potential drug-drug interaction
467 with estrogen; rather, they may impart thrombosis-modulating effects independently. However,
468 several antiviral, antibiotic, and immunomodulatory drugs, as well as some neuromodulators,
469 were effective only in one type of thrombosis. PPIs and RTK/VEGFR inhibitors showed
470 antithrombotic effects in preventing estrogen-induced thrombosis but exhibited prothrombotic
471 profiles in the absence of mestranol, suggesting a potential competition taking place between
472 these drugs and estrogens. The contrasting activities of esomeprazole and omeprazole might be
473 explained by the higher bioavailability of the former, as opposed to the latter which is a racemic
474 mixture that includes esomeprazole.[47]

475 Utilizing both estrogen-induced and genetic thrombosis in zebrafish, we identified
476 distinctive features between these models. This supports the notion that there may be further
477 distinctions both within and between inherited and some forms of acquired thrombosis, and how
478 they arise. Recent studies suggest the possibility that estrogen-induced thrombosis might consist
479 of transglutaminase cross-linked fibrin(ogen), instead of fibrin. Transglutaminases are membrane-
480 bound proteins involved in cross-linking extracellular matrix components. They are involved in
481 numerous physiological and pathological processes including wound healing and angiogenesis.[48-
482 50] Lisetto and colleagues reported a high degree of conservation between zebrafish and human
483 transglutaminases. They observed speckled localization of transglutaminase activity, particularly
484 around the vasculature by the caudal veins of the tail region. Interestingly, this pattern coincides
485 with the distribution of estrogen-induced thrombosis that we observe.[51] It has long been known
486 that non-factor XIII transglutaminases crosslink fibrinogen ex vivo,[52] and a recent study
487 demonstrated that tissue transglutaminase modifies FXIII-directed fibrin(ogen) cross-linking in
488 vivo.[53]

489 In parallel with measuring thrombosis level changes and evaluating potential therapeutic
490 efficacy, we tested the candidate drugs' effects on hemostasis and found that clotting times

491 closely aligned with controls. In contrast, standard anticoagulants resulted in significant delays in
492 occlusion, as we have previously shown, without mitigating estrogen-induced thrombosis.[22,45]
493 These findings suggest that the candidate drugs could potentially modulate clot formation in the
494 acquired model without the hemostatic risk inherent in approved anticoagulants.

495 Given the lack of efficacy in the estrogen-induced thrombosis model, we investigated the
496 possibility of a mestranol interaction that might reduce the anticoagulant effects of standard blood
497 thinners. Our simultaneous assessments of induced thrombosis and time to occlusion indicated
498 that the limited efficacy was likely not due to such interactions, as anticoagulant treatments still
499 resulted in significant delays in occlusion. It was surprising to see that mestranol combined with
500 the DOACs led to further increases in time to occlusion, suggesting a potentiation of the
501 anticoagulant effect. This phenomenon is reminiscent of our past work on antithrombin deficiency
502 in zebrafish.[22] We found that loss of antithrombin, which should result in a prothrombotic state,
503 led to a delayed hemostatic profile, which was rescued by infusion of fibrinogen.[22] This suggested
504 a disseminated intravascular coagulation-like state, where there is an overall consumption of
505 fibrinogen due to unchecked thrombin activity, although the effects we observe in this study are
506 milder.

507 Though it was not as severe, a degree of bleeding risk was observed across some of the
508 candidate drugs as evidenced by delayed occlusion in combination with estrogens, including
509 liothyronine, mifepristone, RTK inhibitors, neuromodulators like etomidate and AM-251, and the
510 immunomodulatory drug laquinimod. In contrast, exemestane, L-thyroxine, and PPIs like
511 esomeprazole and dexlansoprazole exhibited normal occlusion. These findings highlight potential
512 increased hemostatic risks for patients with elevated estrogen who are using the former group of
513 agents.

514 Thyroid hormone medications demonstrated promising nanomolar efficacy and limited
515 hemostatic risks. Interestingly, L-thyronine, which is structurally similar to thyroid hormone but
516 does not have iodine, or L-thyrosine that lacks the terminal phenol ring present in thyroid hormone,

517 did not exhibit the antithrombotic effect. These structural nuances suggest that the presence of
518 iodine on the terminal ring, rather than the inner one, is crucial in the observed antithrombotic
519 effects. Additionally, the carboxyl chain in tiratricol, as opposed to the amine moiety in thyroid
520 hormone, enhances the potency of the former. We used CRISPR-mediated knockdown to test if
521 the thyroid hormone effect is on-target through a known receptor. Zebrafish have two paralogs
522 for the human thyroid receptor alpha (*THRA*), *thraa* and *thrab*, and we found that *thrab* is the
523 major paralog required for antithrombotic activity. This suggests that THRA modulation could be
524 an important target in humans for improving therapeutic strategies in estrogen-induced
525 thrombosis. Both estrogen and thyroid signaling are known to influence thromboembolism.[25-28,54-
526 58] Thus, thyroid hormone signaling could offer an alternative route for thrombosis prevention.
527 Further studies are warranted to elucidate the exact mechanisms underlying its involvement in
528 mediating thrombosis. Knockouts could be also employed to investigate the marginal delays in
529 TTO values that persisted after *thr* knockdown, indicating residual receptor activity from existing
530 THR levels in the embryos.

531 While our experimental studies suggest that thyroid medications may prevent thrombosis,
532 EHR data indicate higher thrombosis rates among individuals using thyroid agonists with oral
533 contraceptives, compared to those not using them concurrently. This discrepancy might be
534 explained by the low baseline incidence of thrombosis observed in such individuals (27 cases
535 among 2,101 patients), non-reported comorbidities, and medications not included in our analysis
536 leading to alterations in hemostatic balance that affect their risk. Additionally, review of the
537 literature indicates a higher prevalence of thrombosis when there is an imbalance or pathology in
538 the thyroid system.[25-28] However, it is unclear whether thrombosis occurs prior to treatment or
539 persists even when on thyroid medications. Hormonal contraceptives can increase thyroid
540 stimulating hormone (TSH) and thyroxine binding globulin without changing free thyroxine
541 levels.[29] In contrast, excess thyroid hormone exposures in our studies mimic a hyperthyroid state
542 which could be a factor for preventing estrogen-induced thrombosis. The EHR data do not

543 explicitly indicate whether the patients on thyroid hormone therapies are in a hyper- or euthyroid
544 state by the time of a thrombotic event. Moreover, other health conditions (e.g., cancer),
545 medications, or genetic predisposition, could lead to a pro-thrombotic state and/or affect
546 interactions with thyroid medications and estrogens. This creates a complex interplay that is much
547 less controlled than our zebrafish models. To gain a better understanding, analysis using a larger
548 population size and higher-resolution data from multicenter datasets is necessary. Further
549 zebrafish mechanistic studies are also essential to clarify causal links, as would a prospective
550 human study controlling and accounting for health status, thyroid medication use, oral
551 contraceptive use, and hormone levels.

552 In summary, we observed distinct drug-response profiles and possible pathological
553 differences between genetic and acquired thrombosis. Notably, PPIs, RTK/VEGFR inhibitors, and
554 hormone-related compounds, emerged with efficacy against estrogen-induced thrombosis.
555 Among them, thyroid hormones showed on-target effects with nanomolar potency in vivo, but
556 observational EHR data linking hormonal contraceptive-related thrombosis and thyroid imbalance
557 are still preliminary. Nonetheless, THR signaling stands out as a promising target for future
558 mechanistic studies and drug development.

559

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565 statistical analyses. Additionally, we extend our gratitude to Dr. Jasmine Luzum for her expert
566 insights on the precision medicine landscape for VTE therapies and management, and Donald
567 McDonnell for helpful discussions.

568

Authorship Contributions

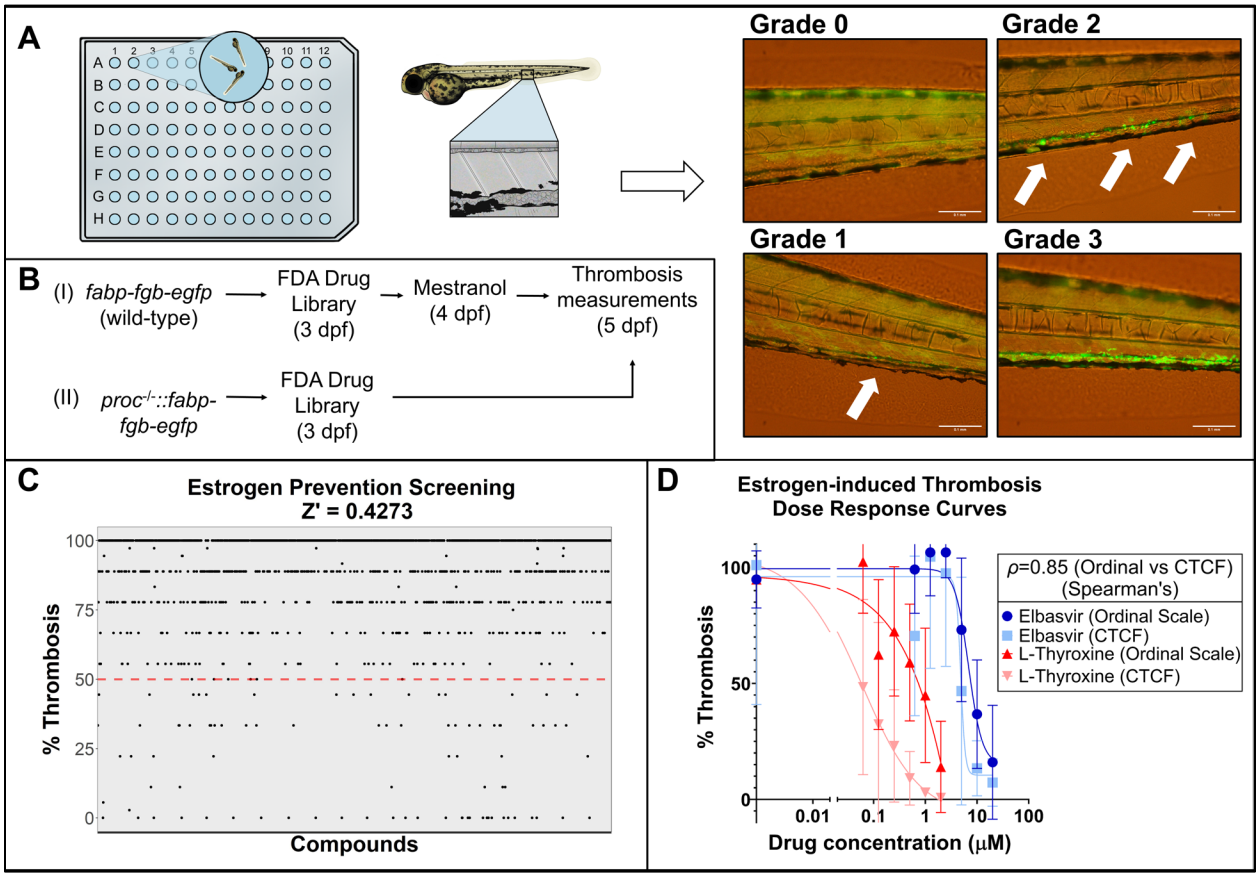
M.Y. and H.S. designed and performed research, analyzed data, and wrote the manuscript. M.Y. and H.S. are co–first authors. The order of co–first authorship was determined based on the overall scope and breadth of their respective contributions to the study. M.Y. led the comprehensive drug screening efforts and coordinated the integration of experimental work across study segments. H.S. directed and conducted the thyroid hormone-related investigations and contributed substantially to manuscript development. J.K.L. and C.J.H. designed and performed research, and analyzed data, J.X. and D.W. performed research and analyzed data, A.C.F. and K.M.S. performed research, D.A.H., M.C.C., and J.R. supervised research and edited the manuscript, and J.A.S. designed and supervised research, and edited the manuscript.

Conflict of interest Disclosures

J.A.S. has been a consultant for Sanofi, NovoNordisk, Biomarin, Takeda, Pfizer, Genentech, CSL Behring, and Medexus. The other authors declare no relevant conflicts of interest.

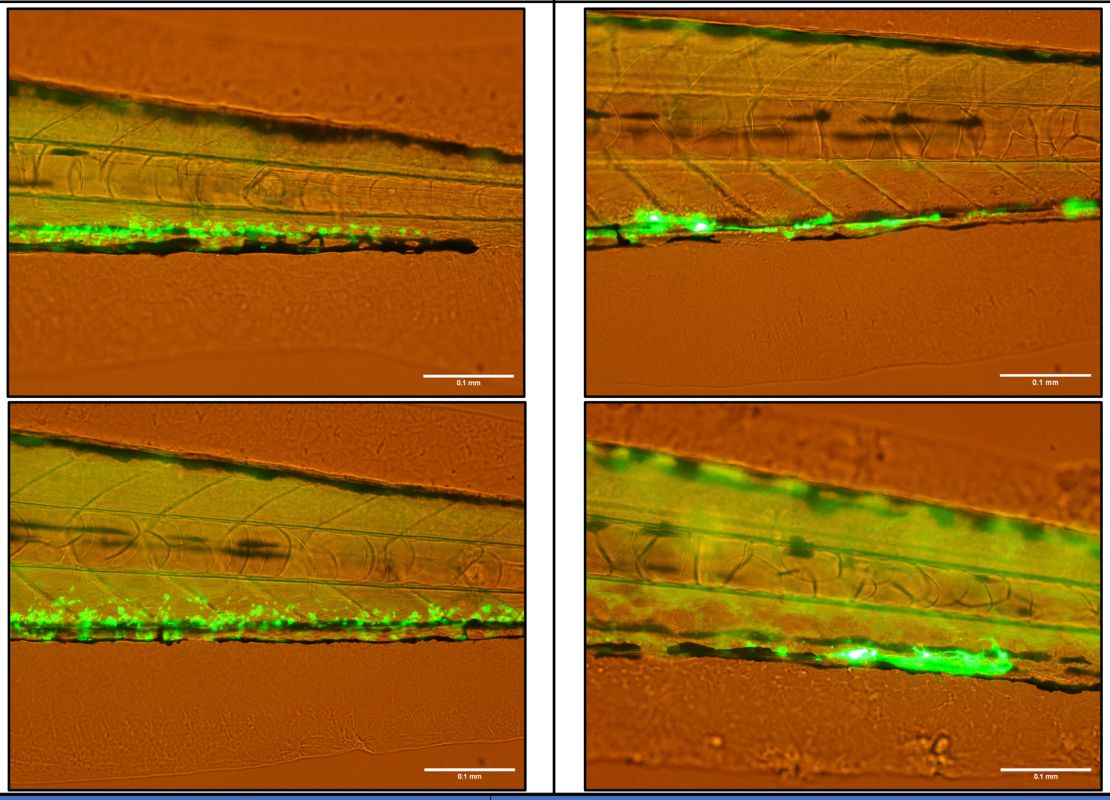
583 **FIGURES**

584



585

586 **Figure 1: Drug library screening in zebrafish models of thrombosis identifies potential anti-**
587 **thrombotic agents.** (A) Transgenic zebrafish models expressing eGFP-tagged fibrinogen were
588 employed to semi-quantitatively assess thrombosis levels in the caudal veins using an ordinal
589 scale from grades 0 to 3 (arrows indicate areas of thrombosis). Imaging and drug exposure
590 protocols were standardized using a 96-well plate format, with 3 embryos per well. (B) Both
591 acquired (estrogen-induced) and genetic (*proc* mutant/spontaneous) thrombosis models were
592 subjected to drug treatments at 3 dpf. Thrombosis levels were subsequently graded at 5 dpf. For
593 the acquired model, thrombosis was triggered by exposure to 25 μM mestranol at 4 dpf. (C)
594 Screening in the acquired model evaluated 1,600 compounds at a concentration of 25 μM. The
595 mean thrombosis level for each treatment (according to the grading scale in (A)) is depicted as a
596 percentage change. An arbitrary threshold was set at 50% reduction which identified 117 hits with
597 a *Z'* score of 0.4273. (D) Representative dose-response curves for the acquired model.
598 Thrombosis scores were obtained for each treatment quantitatively (CTCF) and semi-
599 quantitatively (ordinal scale grades 0-3) from the same images. Four-parameter log(agonist) vs.
600 response curves were modeled based on the concentrations of elbasvir and L-thyroxine on the x-
601 axis and thrombosis percentages on the y-axis, demonstrating a close approximation between
602 qualitative and quantitative measurements. Spearman's correlation coefficient (ρ) for each group
603 was 0.85. Error bars represent 95% confidence intervals. All data were collected by an observer
604 blinded to the condition.



B

Metrics	Machine Learning Models	
	CNN	SVM
Sensitivity	0.90	0.97
Specificity	0.89	0.69
Accuracy	0.90	0.83
Area Under Curve (AUC)	0.90	0.83

Figure 2: Image analyses of the acquired and genetic models of thrombosis identify distinct clotting patterns. (A) Speckled pattern of thrombus distribution was observed in the images obtained from the acquired model, while a sprouting pattern with denser fluorescence signals was noted in the genetic model. (B) To test these differences, we developed convolutional neural networks (CNN) and support vector machine (SVM) models that distinguished between the two based on fluorescence images, achieving a high degree of sensitivity and accuracy using images from six independent experiments. Scale bars, 100 μm. All data were analyzed by an observer blinded to the condition.

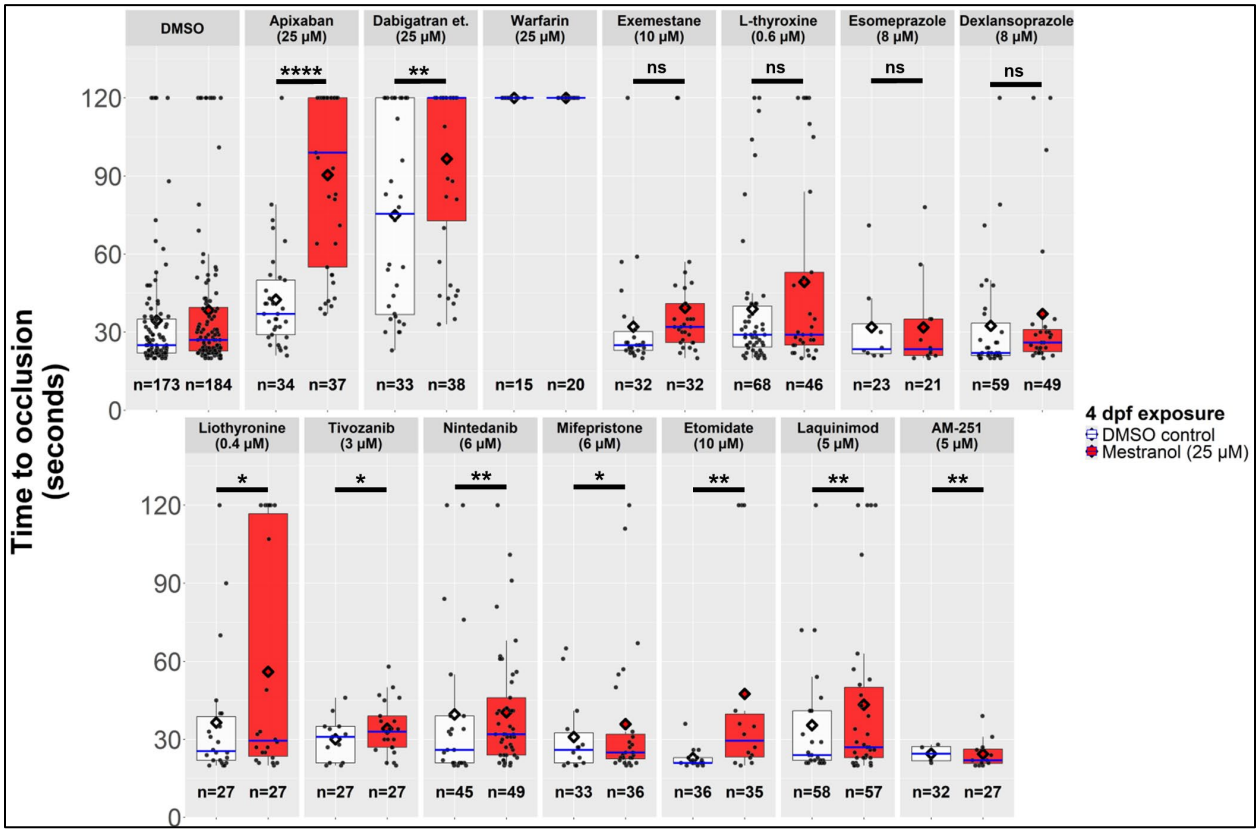


Figure 3: Candidate compounds exhibit reduced hemostatic risk compared to standard anticoagulants. Time to occlusion (TTO) values for candidate compounds were assessed by laser-mediated endothelial injury and consistent with DMSO control, indicating a lower hemostatic risk compared to standard-of-care therapies. Some candidates increased occlusion times in the presence of estrogens, but this effect was less pronounced than that observed with standard anticoagulants, both with and without estrogens. In the box plots, median values are indicated in blue, and mean TTO values for each treatment are marked with a diamond. Statistical analyses were performed using multivariate Cox proportional hazards regression models with the *survival* and *emmeans* libraries in R (v4.3.3). ****: $p<0.0001$, **: $p<0.01$, *: $p<0.05$, ns: not significant. All data were collected by an observer blinded to the condition.

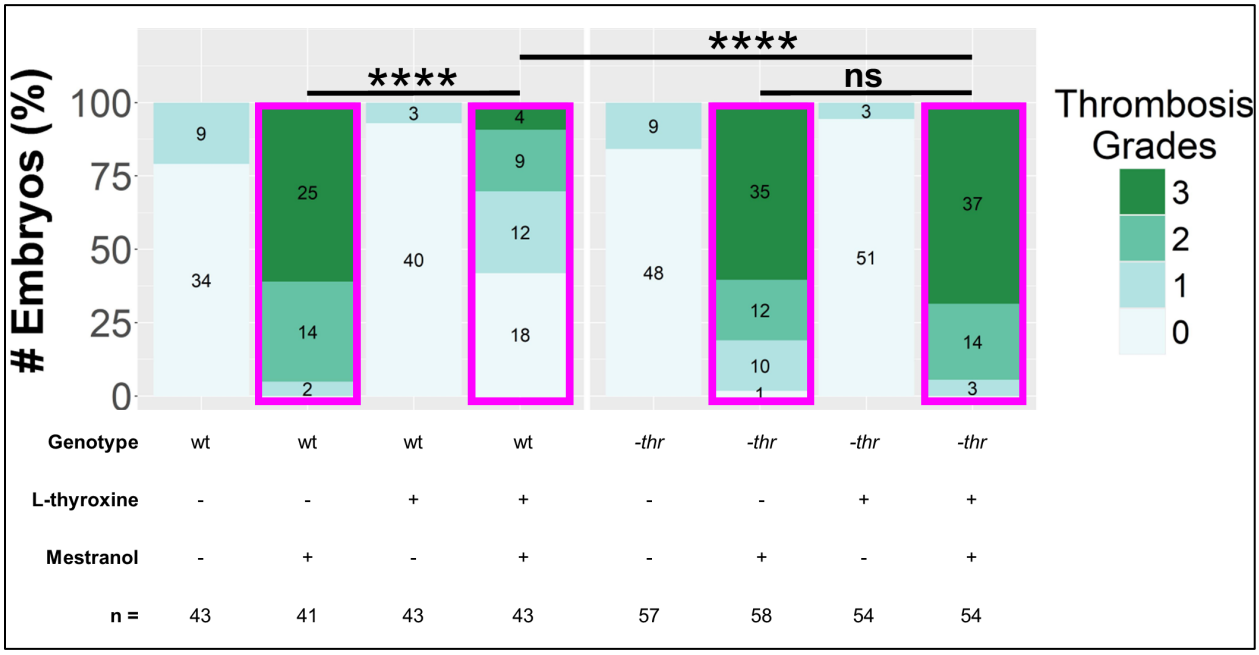


Figure 4: The antithrombotic effect of thyroid hormone is mediated through known receptors. CRISPR sgRNA/Cas9 complexes were injected into single-cell embryos that were then treated with L-thyroxine (0.6 μM) at 3 dpf and mestranol (25 μM) at 4 dpf. Statistical analyses were performed using proportional odds logistic regression (polr) models with MASS and *emmeans* libraries in R (v4.3.3). ****: $p < 0.0001$, ns: not significant. wt: wild-type (uninjected *fabp-fgb-egfp* control group), *-thr*: combined knockdown of all thyroid hormone receptor paralogs *thraa*, *thrab*, and *thrb*. Magenta borders indicate mestranol treated groups. All data were collected by an observer blinded to the condition. Sample sizes for each group, corresponding to the thrombosis grades, are indicated within their respective stack.

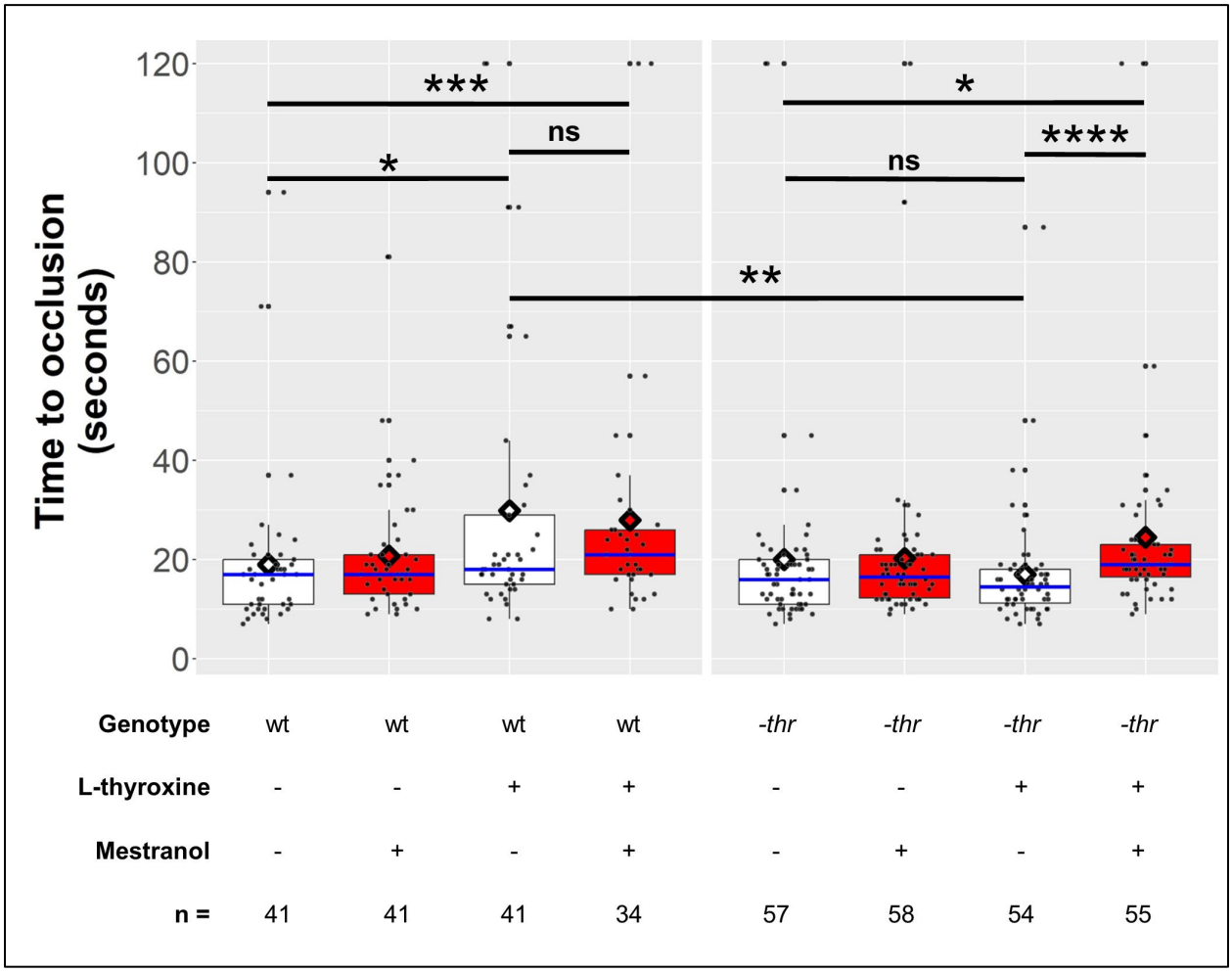


Figure 5: Thyroid hormone modulates hemostasis through known receptors in the estrogen-induced thrombosis model. CRISPR sgRNA/Cas9 complexes were injected into single-cell embryos that were then treated with L-thyroxine (0.6 μM) at 3 dpf, and mestranol (25 μM) at 4 dpf, followed by assessment of laser-mediated endothelial injury at 5 dpf. Increased TTO observed in wild-type embryos treated with thyroid hormone was diminished upon *thr* knockdown, indicating involvement of thyroid receptor signaling. Increased TTO levels were still present in the L-thyroxine-mestranol combination groups. ****: $p<0.0001$, ***: $p<0.001$, **: $p<0.01$, *: $p<0.05$, ns: not significant. wt: wild-type (uninjected *fabp-fgb-egfp* control group), *-thr*: combined knockdown of all thyroid hormone receptor paralogs *thraa*, *thrab*, and *thrb*. All data were collected by an observer blinded to the condition.

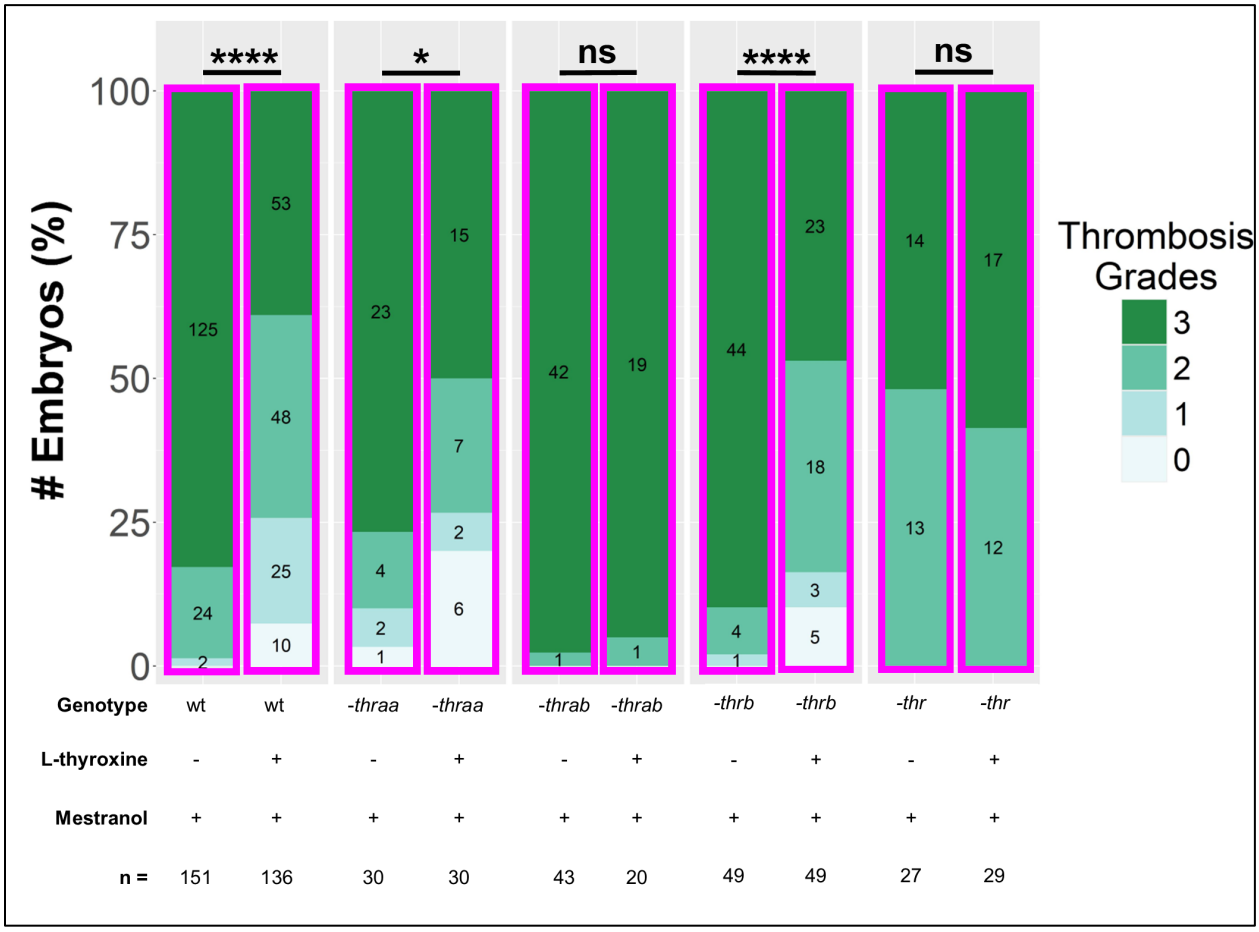


Figure 6: *thrab* receptor modulates the antithrombotic effect of thyroid hormone. After CRISPR-mediated individual knockdown of each canonical thyroid hormone receptor (-*thraa*, -*thrab*, and -*thrb*) or combination (-*thr*), embryos were co-treated with L-thyroxine (3 μM) and mestranol (25 μM) at 4 dpf and evaluated 24 hours later. Magenta borders indicate mestranol treated groups. ****: $p < 0.0001$, *: $p < 0.05$, ns: not significant. All data were collected by an observer blinded to the condition. Sample sizes for each group, corresponding to the thrombosis grades, are indicated within their respective stack.

658 **TABLES**

Compounds	IC_{30}		Compounds	IC_{30}	
	Acquired	Genetic		Acquired	Genetic
AM-251	6.72	12.87	Exemestane	2.95	13.72
Meclofenoxate	6.03	No effect	Mestranol*	8.47#	11.91#
Loxapine	19.4#	No effect#	1-850	6.07	No effect
Lofepramine	6.04	No effect	Pantoprazole	14.27	No effect
Etomidate	23.08	18.94	Dexlansoprazole	10.69	5.19
Laquinimod	1.43	10.01	Omeprazole	14.57	No effect
Bendazac	1.51	No effect	Esomeprazole*	8.38	5.16
Tiaprofenic acid	2.63	No effect	SCH28080	6.11	No effect
Buparvaquone	No effect	0.69	Ilaprazole	14.79	No effect
Edaravone	No effect	9.12	Elbasvir	1.35	No effect
Malotilate	No effect	24.05	Thiabendazole	No effect	14.34
Belinostat	47.45	No effect	Nalidixic acid	No effect	6.34
Diphenylcyclopropenone	7.89	No effect	Indole-3-carboxylic acid	7.89	No effect
Tivozanib*	0.67	1.68	Benvitimod	2.82	No effect
Nintedanib*	6.93	4.06	Phenindione	42.82	9.64
L-Thyroxine	0.2	0.15	Apixaban	No effect	No effect
Liothyronine	0.28	0.34	Warfarin	No effect	0.02
Mifepristone*	7.44	10.10	Dabigatran etexilate	No effect	4.58
			Rivaroxaban	No effect	No effect

659 **Table 1: IC_{30} values for the candidate compounds tested on acquired (estrogen-induced)**
660 **and genetic (spontaneous/PC deficient) models of thrombosis.** The values (μM) were
661 obtained from dose response curves. No effect, no IC_{30} detected from the dose response curves.
662 Hits that are not concordant between the models are highlighted in gray. #: IC_{30} values are derived
663 from the procoagulant profiles of the respective drugs in the acquired model with no mestranol
664 treatment. *: compounds with procoagulant effects in the genetic model.

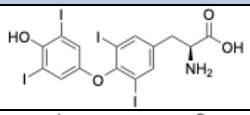
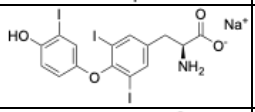
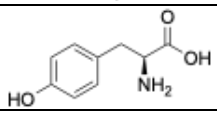
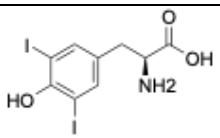
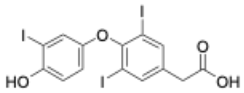
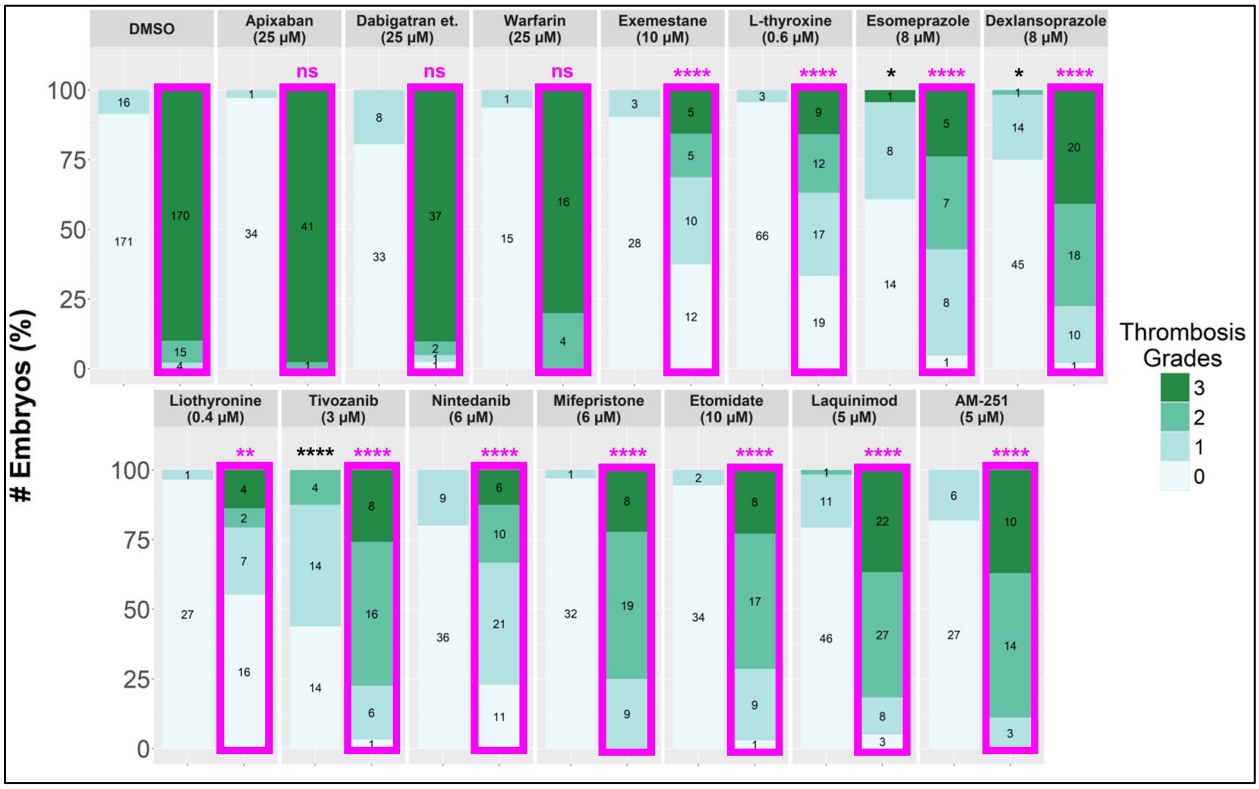
Compounds	Structures	IC_{30} (μM)		IC_{50} (μM)	
		Acquired	Genetic	Acquired	Genetic
L-thyroxine		0.2	0.15	0.83	0.32
Liothyronine		0.28	0.34	0.36	0.8
L-tyrosine		No effect	No effect	No effect	No effect
L-thyronine		No effect	No effect	No effect	No effect
3,5-diiodo-L-tyrosine		No effect	No effect	No effect	No effect
3,3',5-triiodothyroacetic acid (tiratricol)		0.09	<0.1	0.24	<0.1

Table 2: IC_{30} and IC_{50} values for thyroid hormone-related structures, highlighting the importance of iodine groups in antithrombotic effects. #, IC_{30}/IC_{50} values are derived from the procoagulant profiles in the acquired model with no additional estrogen treatments by 4 dpf. *, compounds with procoagulant effects in the genetic model.

University of Michigan Data			
	HC alone	**HC with Thyroid Medication**	**Thyroid Medication Alone**
All Patients			
Thrombosis	321	27	260
All	47,710	2,101	30,921
Percentage	**0.67%**	**1.27%**	**0.83%**
Odds Ratio	**1.9 (1.29-2.82)**		
		1.5 (1.04-2.26)	
Duration			
Days of HC	674 (668-681)	557 (528-585)	
Days of Thyroid Medication		557 (528-585)	847 (833 - 860)

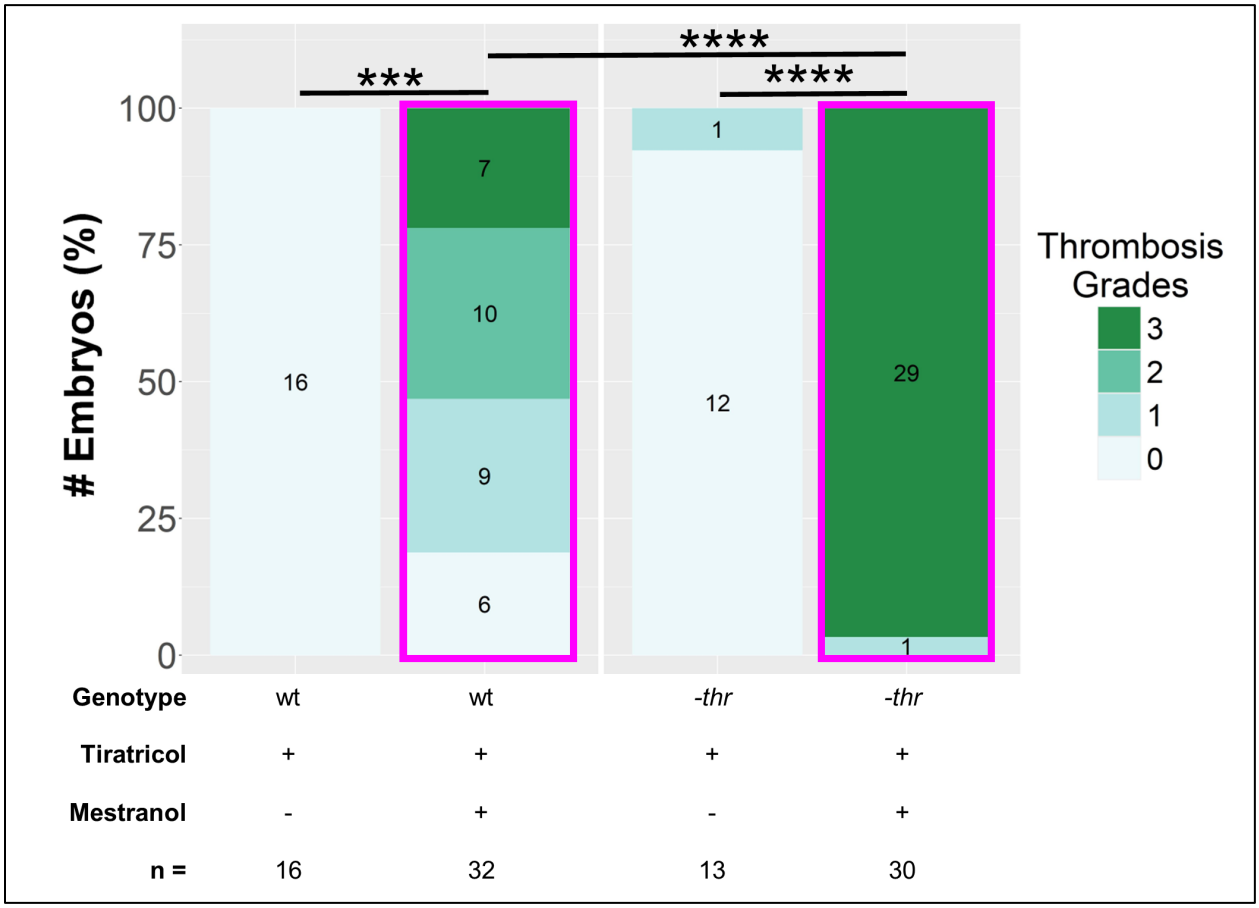
Table 3: Significant increase in thrombosis rates for the combination HC and thyroid medication group. Patients with reported concordant use of HC and thyroid medications have statistically significant increase in thrombosis rates.

673 **SUPPLEMENTAL DATA**

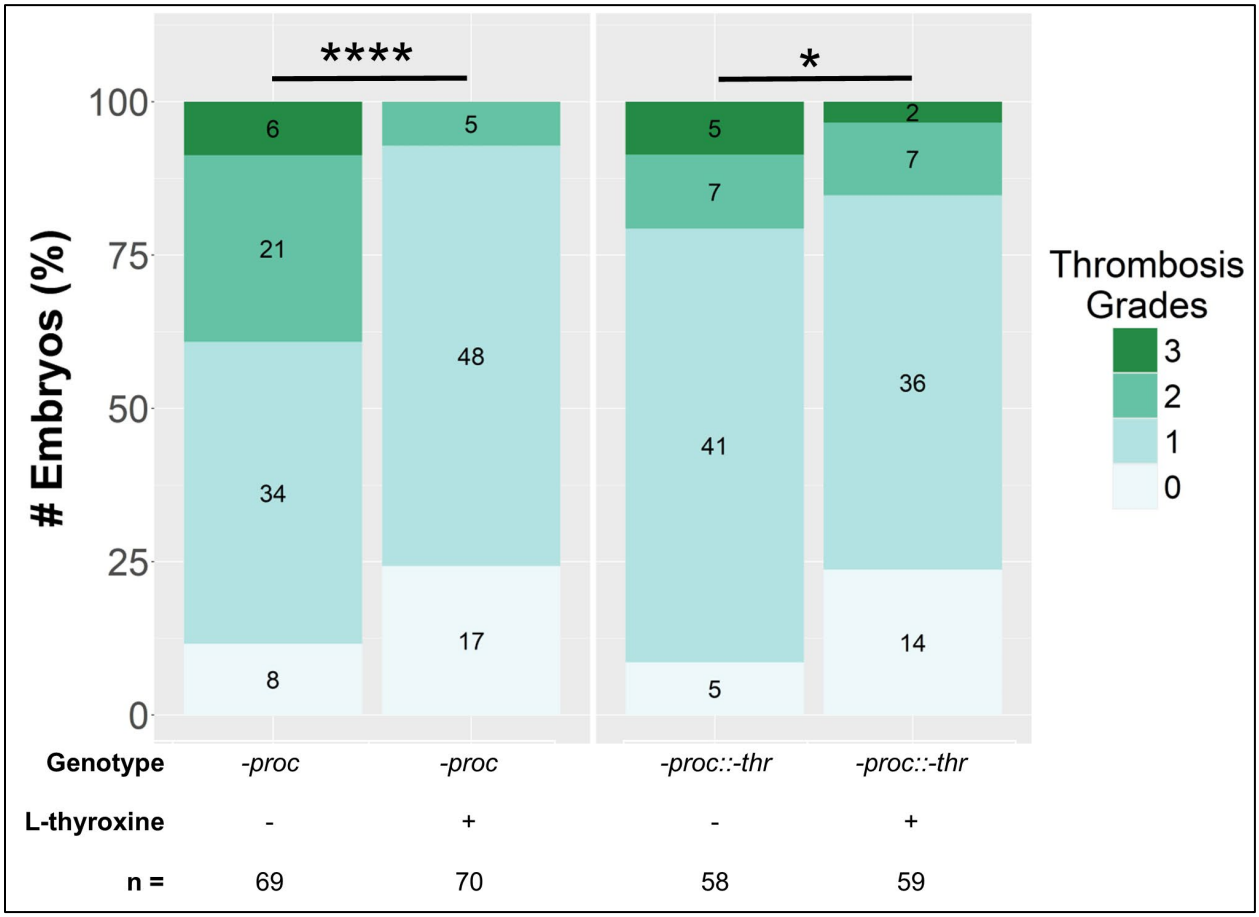


674

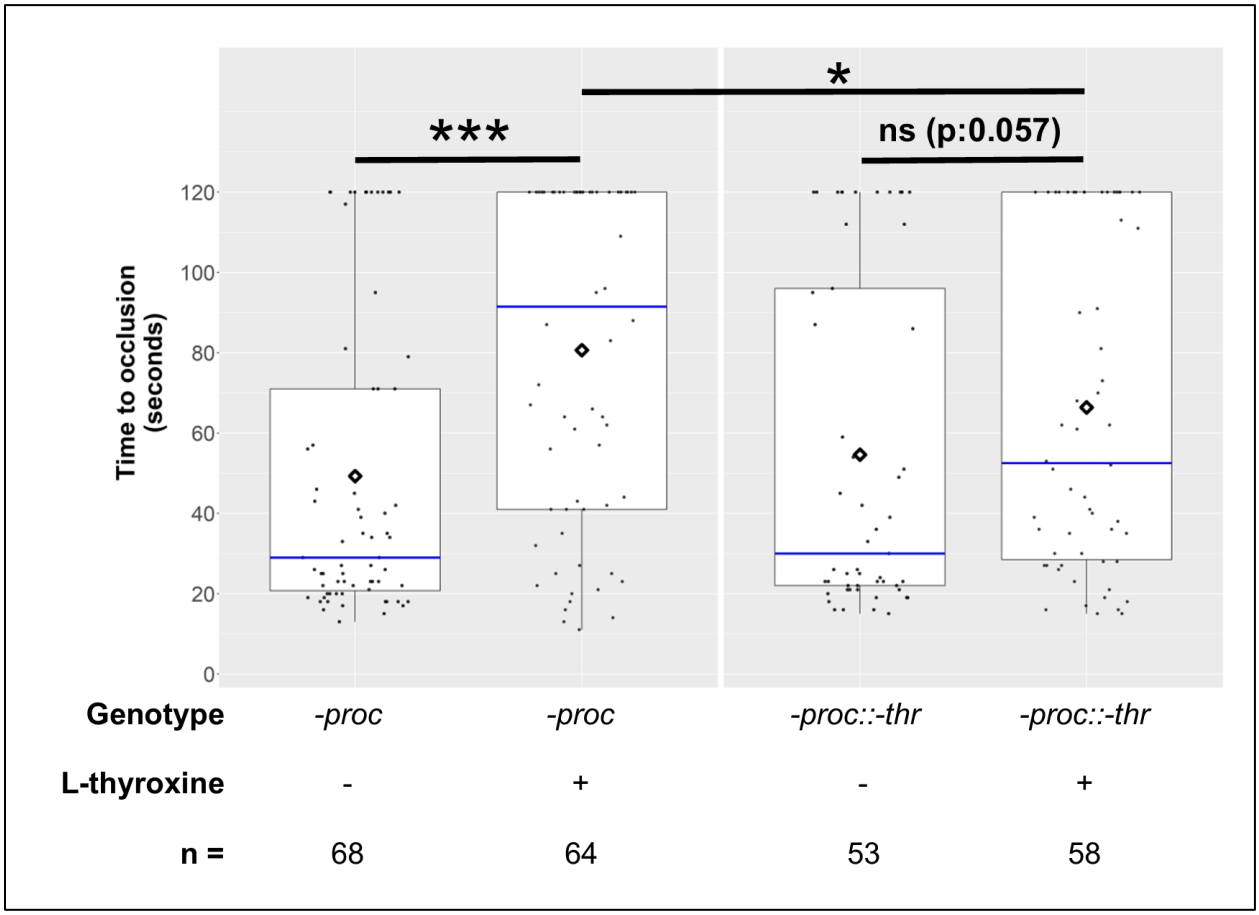
675 **Supplemental Figure 1: Thrombosis grades confirm effective drug concentrations in**
676 **relation to TTO levels in Figure 3.** The concentrations of candidate drugs used in the laser injury
677 experiments in Figure 3 were confirmed to be within an effective range for modulating thrombosis.
678 Thrombosis grades were collected simultaneously with the TTO evaluations from the same
679 embryos. Magenta borders indicate treatments that included mestranol (25 µM) exposure. ****:
680 $p<0.0001$, **: $p<0.01$, *: $p<0.05$, ns: not significant (p-values are calculated against DMSO and
681 estrogen controls, black and magenta colored asterisks, respectively). Sample sizes for each
682 group, corresponding to the thrombosis grades, are indicated within their respective stack.



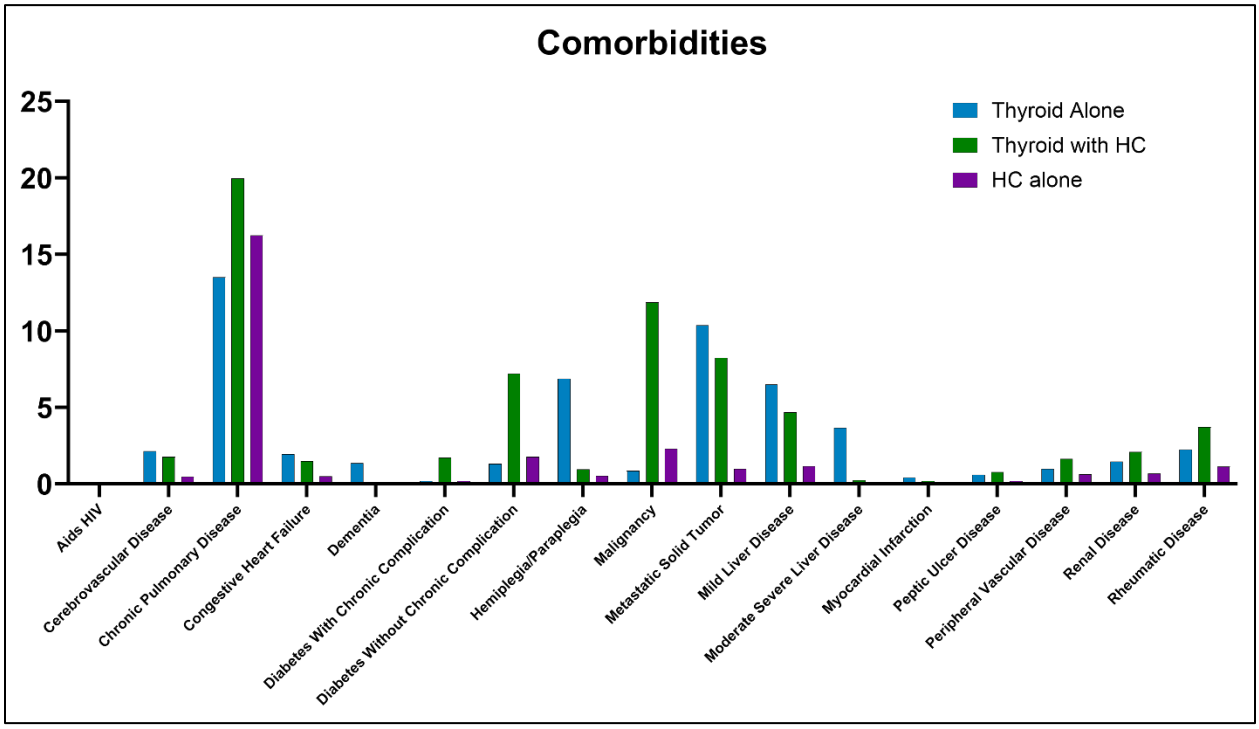
Supplemental Figure 2: The antithrombotic effect of thyroid hormone analog (tiratricol) is mediated through known receptors. CRISPR sgRNA/Cas9 complexes were injected into single-cell embryos that were then treated with tiratricol (0.1 μM) at 3 dpf and mestranol (25 μM) at 4 dpf. Statistical analyses were performed using proportional odds logistic regression (polr) models with MASS and *emmeans* libraries in R (v4.3.3). To prevent infinitesimal values that would be generated by the regression models, each thrombosis grade group was incremented by one count across all treatment groups. ****: $p < 0.0001$, ***: $p < 0.001$. wt: wild-type (uninjected *fabp-fgb-egfp* control group), *-thr*: combined knockdown of all thyroid hormone receptor paralogs *thraa*, *thrab*, and *thrb*. Magenta borders indicate mestranol treated groups. All data were collected by an observer blinded to the condition. Sample sizes for each group, corresponding to the thrombosis grades, are indicated within their respective stack.



Supplemental Figure 3: The antithrombotic effect of thyroid hormone is mediated through known receptors in the genetic VTE model. CRISPR sgRNA/Cas9 complexes were injected into single-cell embryos that were then treated with L-thyroxine (0.6 μM) at 3 dpf and observed at 5 dpf. Thrombosis grades were collected simultaneously with the TTO evaluations, ensuring consistent sample use as presented in **Supplemental Figure 4**. Statistical analyses were performed using proportional odds logistic regression (*polr*) models with MASS and *emmeans* libraries in R (v4.3.3). ****: $p < 0.0001$, *: $p < 0.05$. *-proc*: genetic thrombosis line (*proc*$^{-/-}$*::fabp-fgb-egfp* control group), *-thr*: combined knockdown of all thyroid hormone receptor paralogs *thraa*, *thrab*, and *thrb*. All data were collected by an observer blinded to the condition. Sample sizes for each group, corresponding to the thrombosis grades, are indicated within their respective stack.



Supplemental Figure 4: Thyroid hormone modulates hemostasis through known receptors in the genetic model. Following thrombosis grading (**Supplemental Figure 3**), viable embryos underwent laser-mediated endothelial injury at 5 dpf. Increased TTO observed in genetic thrombosis treated with thyroid hormone was diminished upon *thr* knockdown, supporting involvement of thyroid receptor signaling. A marginal TTO delay in the -*thr* group with L-thyroxine treatment suggests residual receptor activity across *thr* knockdown samples. ***: $p<0.001$, *: $p<0.05$, ns: not significant. -*proc* (genetic thrombosis line: *proc*$^{-/-}$*::fabp-fgb-egfp* control group), -*thr*, combined knockdown of all thyroid hormone receptor paralogs *thraa*, *thrab*, and *thrb*. All data were collected by an observer blinded to the condition.



Supplemental Figure 5: Increased incidence of comorbidities in the EHR dataset for the HC with thyroid medication and thyroid medication alone groups. Notably, the HC with thyroid medication group had higher rates of chronic pulmonary disease, malignancy, and diabetes. The thyroid medication alone group showed increased incidence of mild liver disease, hemiplegia, and metastatic solid tumors, otherwise there were no statistical differences between the groups in specific comorbidities.

Plate No & Well Position	Drug Name	Plate No & Well Position	Drug Name	Plate No & Well Position	Drug Name
1_8A	Everolimus	7_3D	Methotrexate	13_4H	p-Toluic acid
1_8H	Belinostat	7_5D	Amenamevir	13_5B	Alosetron (Hydrochloride)
1_9A	Sorafenib Tosylate	7_6F	Thalidomide	13_7G	Crizotinib (hydrochloride)
1_10A	Rapamycin	7_8G	Entacapone	13_8D	Crizotinib
1_10D	Telaprevir	7_10C	Rolapitant	13_11G	Desfesoterodine
1_10F	Rilmenidine hemifumarate	8_2F	Opicapone	14_6D	Liothyronine (sodium)
1_11A	Ilaprazole	8_4H	Avermectin B1	14_7E	Malotilate
2_5B	Daclatasvir dihydrochloride	8_7B	Repaglinide	14_7G	Cefotaxime (sodium salt)
2_5E	Dapagliflozin	9_11D	Elbasvir	14_8A	Liothyronine
2_7A	Tiaprofenic acid	10_2D	Iguratimod	14_9D	Sapropterin (dihydrochloride)
2_8E	Daclatasvir	10_5D	Fluralaner	14_10C	Docetaxel
2_10E	Niraparib hydrochloride	10_6D	Idarubicin (hydrochloride)	15_5H	Bendamustine (hydrochloride)
2_11E	Neflamapimod	10_9H	Ziprasidone (hydrochloride monohydrate)	15_7D	Acarbose
3_2D	Promestriene	10_11D	Racecadotril	15_7F	Omeprazole (sodium)
3_2E	Nintedanib esylate	11_4G	Pantoprazole (sodium hydrate)	15_10C	Tropisetron
3_4D	Valemetostat (tosylate)	11_3A	Meclofenoxate (hydrochloride)	15_10D	Etomidate (hydrochloride)
3_6C	Lenvatinib	11_4G	Pantoprazole (sodium hydrate)	16_3D	Milnacipran ((1S-cis) hydrochloride)
3_8G	Delamanid	11_6A	Atosiban (acetate)	16_5B	Detomidine (hydrochloride)
3_8H	Tivozanib	11_8A	Ampiroxicam	16_6A	Lithocholic acid
3_9D	Ripretinib	11_10E	Parecoxib (Sodium)	16_10G	Chlorothiazide
3_11G	Enarodustat	11_11A	Boldenone Undecylenate	17_8D	Oxytetracycline
4_6A	Velpatasvir	11_11H	Bendazac	18_6D	Liranaftate
4_7C	Idelalisib	11_5D	Pantoprazole (sodium)	18_10B	Bupivacaine (hydrochloride)
4_8A	Bempedoic acid	11_4G	Pantoprazole (sodium hydrate)	18_10C	Nicorandil
4_9D	Dantrolene (sodium hemiheptahydrate)	12_2F	L-Thyroxine (sodium salt)	19_2A	Loperamide (hydrochloride)
4_11B	Rifamycin S	12_5A	Conivaptan (hydrochloride)	19_2H	Clomipramine (hydrochloride)
4_11G	Laquinimod	12_5E	Dalbavancin (hydrochloride)	19_3H	Phenoxybenzamine (hydrochloride)
5_2E	Fostamatinib (disodium hexahydrate)	12_6D	L-Thyroxine	19_5F	Ciclopirox (olamine)
5_5A	Lomustine	12_7A	Ornidazole (Levo-)	19_7C	Ornidazole
5_5C	Deflazacort	12_7G	Pleconaril	19_9B	Niclosamide
5_5F	Cladribine	12_9A	Glecaprevir	19_11B	Domperidone
5_6A	Cytarabine	12_9C	Filgotinib	20_3D	Sodium nitroprusside
5_6D	Cyproterone acetate	12_10E	L-Hydroxyproline	20_3H	Naftifine (hydrochloride)
5_7B	Rupatadine (Fumarate)	12_10G	trans-Tranilast	20_4H	Ampicillin (sodium)
5_8E	Exemestane	12_11A	Indole-3-carboxylic acid	20_5G	Tafluprost
5_10A	Anastrozole	12_11B	Quercetin	20_7D	Metformin
5_11B	Telotristat ethyl	12_11G	Hemin	20_7H	Atorvastatin
6_9H	Pirarubicin (Hydrochloride)	13_2F	Ammonium glycyrrhizinate	20_9B	Altrenogest
6_10F	Isocarboxazid	13_4D	Nintedanib	20_11G	Methylthiouracil

Supplemental Table 1: MedChemExpress HY-L066 Library acquired thrombosis model hits (n=117)

Compounds	Company - Catalog No	Compounds	Company - Catalog No
AM-251	Cayman Chemical - 71670	1-850	Cayman Chemical - 17248
Meclofenoxate (hydrochloride)	Cayman Chemical - 28283	Pantoprazole sodium	Sigma - PHR1604
Loxapine succinate	Sigma - L106	Dexlansoprazole	Sigma - SML2624
Lofepramine hydrochloride	Sigma - BP697	Omeprazole	Sigma - PHR1059
Etomidate	Selleckchem - S1329	Esomeprazole magnesium	Sigma - PHR1585
Laquinimod	Sigma - SML2504	SCH28080	MedChemExpress - HY-103261
Bendazac	Selleckchem - S5361	Ilaprazole	Sigma - SML2322
Diphenylcyclopropenone	Sigma - 177377	Elbasvir	Selleckchem - S5652
Belinostat	MedChemExpress - HY-10225	Benvitimod	Sigma - SML3430
Tivozanib	Sigma - SML3132	Indole-3-carboxylic acid	Sigma - 284734
Nintedanib	Sigma - SML2848	Phenindione	Selleckchem - S1921
L-Thyroxine	Sigma - T1775	Apixaban	Sigma - SML3285
Liothyronine	MedChemExpress - HY-113082	Warfarin	Sigma – A2250
Mifepristone M8046	Sigma - M8046	Dabigatran etexilate	Sigma - SML2351
Exemestane	Sigma - PHR1634	Rivaroxaban	Sigma - SML2844
Mestranol*	Sigma - 855871		

Supplemental Table 2: Validated hit compounds

***thr* paralog**	**Exon**	**sgRNA sequence**
thraa	4	AGAUAACUGGGAACGUACCC
thrab	5	CAUCCUUCUCCAGAUAGCUG
thrb	4	UCAUCCUUGUCCAGGUAACU

***thr* paralog**	**Forward primer**	**Reverse primer**	**Amplicon size (base pairs)**
thraa Exon 4	CATGTGATGCAGCGGTAATGA	TGCAGTCGCCTATTCTCTCCT	140
thrab Exon 5	TGTGCACAAGCGATAGCAGA	GCATGGCAGGGTACATCC	140
thrb Exon 4	CTCACATGTAATGCAGCGATAGTG	ATGACGAGGTCTGTTGTCTTGG	359

730 **Supplemental Table 3: Sequences of sgRNAs and PCR primers**

Compounds	IC_{30}		IC_{50}	
	Acquired	Genetic	Acquired	Genetic
AM-251	6.72	12.87	No effect	14.02
Meclofenoxate	6.03	No effect	10.69	No effect
Loxapine	19.4#	No effect#	No effect	No effect
Lofepramine	6.04	No effect	No effect	No effect
Etomidate	23.08	18.94	26.91	No effect
Laquinimod	1.43	10.01	2.89	No effect
Bendazac	1.51	No effect	9.36	No effect
Tiaprofenic acid	2.63	No effect	5.13	No effect
Buparvaquone	No effect	0.69	No effect	1.08
Edaravone	No effect	9.12	No effect	13.1
Malotilate	No effect	24.05	No effect	59.99
Belinostat	47.45	No effect	No effect	No effect
Diphenylcyclopropenone	7.89	No effect	No effect	No effect
Tivozanib*	0.67	1.68	3.53	No effect
Nintedanib*	6.93	4.06	9.64	No effect
L-Thyroxine	0.2	0.15	0.83	0.32
Liothyronine	0.28	0.34	0.36	0.8
Mifepristone*	7.44	10.10	11.32	No effect
Exemestane	2.95	13.72	5.71	17.80
Mestranol*	8.47#	11.91#	10.19	15.47
1-850	6.07	No effect	No effect	No effect
Pantoprazole	14.27	No effect	27.73	No effect
Dexlansoprazole	10.69	5.19	13.77	18.80
Omeprazole	14.57	No effect	21.35	No effect
Esomeprazole*	8.38	5.16	10.62	No effect
SCH28080	6.11	No effect	6.62	No effect
Ilaprazole	14.79	No effect	73.45	No effect
Elbasvir	1.35	No effect	1.71	No effect
Thiabendazole	No effect	14.34	No effect	No effect
Nalidixic acid	No effect	6.34	No effect	No effect
Indole-3-carboxylic acid	7.89	No effect	10.48	No effect
Benvitimod	2.82	No effect	4.66	No effect
Phenindione	42.82	9.64	66.04	19.93
Apixaban	No effect	No effect	No effect	No effect
Warfarin	No effect	0.02	No effect	0.05
Dabigatran etexilate	No effect	4.58	No effect	11.72
Rivaroxaban	No effect	No effect	No effect	No effect

731 **Supplemental Table 4: IC_{30} and IC_{50} values for the candidate compounds tested on**
732 **acquired (estrogen-induced) and genetic (spontaneous/PC deficient) models of**
733 **thrombosis.** The values (μM) were obtained from dose response curves. No effect, no IC_{30}/IC_{50}
734 detected from the dose response curves. Hits that are not concordant between the models are
735 highlighted in gray. #, IC_{30}/IC_{50} values are derived from the procoagulant profiles of the respective
736 drugs in the acquired model with no mestranol treatment. *, compounds with procoagulant effects
737 in the genetic model.

Drug	Estimate	Std.error	Statistic	Adj.p.value
DMSO control	0.16	0.11	1.49	0.14
Apixaban (25 μM)	1.26	0.29	4.34	1.40E-05
Dabigatran etexilate (25 μM)	1.04	0.34	3.08	0.002
Warfarin (25 μM)	0.16	0.11	1.49	0.14
Exemestane (10 μM)	0.39	0.26	1.50	0.13
Liothyronine (0.4 μM)	0.68	0.30	2.29	0.02
L-thyroxine (0.6 μM)	0.19	0.20	0.94	0.34
Tivozanib (3 μM)	0.69	0.27	2.51	0.016
Esomeprazole (8 μM)	0.40	0.31	1.30	0.19
Nintedanib (6 μM)	0.66	0.21	3.12	0.0018
Dexlansoprazole (8 μM)	0.18	0.20	0.89	0.37
Mifepristone (6 μM)	0.49	0.24	2.02	0.04
Etomidate (10 μM)	0.81	0.24	3.32	0.0009
Laquinimod (5 μM)	0.54	0.19	2.83	0.0047
AM-251(5 μM)	0.72	0.27	2.72	0.006

Supplemental Table 5: Time to occlusion batch corrected statistics comparing the effect of candidate drugs versus their mestranol combinations in Figure 3. These data show the impact of combining the drugs with mestranol on time to occlusion.

	Drug alone vs DMSO control			
Drug	**Estimate**	**Std.error**	**Statistic**	**Adj.p.value**
Apixaban (25 μM)	-1.21	0.21	-5.79	9.14E-08
Dabigatran etexilate (25 μM)	-1.54	0.24	-6.52	9.24E-10
Warfarin (25 μM)	0	0	inf	inf
Exemestane (10 μM)	-0.33	0.22	-1.51	0.64
Liothyronine (0.4 μM)	-0.36	0.23	-1.54	0.62
L-thyroxine (0.6 μM)	-0.70	0.16	-4.39	0.00014
Tivozanib (3 μM)	0.14	0.22	0.61	0.99
Esomeprazole (8 μM)	0.57	0.24	2.27	0.19
Nintedanib (6 μM)	-0.03	0.19	-0.15	0.99
Dexlansoprazole (8 μM)	-0.09	0.17	-0.54	0.99
Mifepristone (6 μM)	-0.06	0.22	-0.29	0.99
Etomidate (10 μM)	0.50	0.21	2.42	0.14
Laquinimod (5 μM)	0.13	0.17	0.74	0.97
AM-251(5 μM)	1.10	0.21	5.15	3.33E-06

Drug	**Drug + Mestranol vs Mestranol control**			
Apixaban (25 μM)	-2.30	0.26	-8.80	4.03E-14
Dabigatran etexilate (25 μM)	-2.43	0.29	-8.49	4.95E-14
Warfarin (25 μM)	0	0	inf	inf
Exemestane (10 μM)	-0.54	0.22	-2.49	0.12
Liothyronine (0.4 μM)	-0.87	0.25	-3.49	0.006
L-thyroxine (0.6 μM)	-0.73	0.18	-3.96	0.0009
Tivozanib (3 μM)	-0.39	0.22	-1.74	0.49
Esomeprazole (8 μM)	0.33	0.25	1.32	0.75
Nintedanib (6 μM)	-0.52	0.17	-3.03	0.026
Dexlansoprazole (8 μM)	-0.10	0.18	-0.58	0.99
Mifepristone (6 μM)	-0.39	0.21	-1.90	0.39
Etomidate (10 μM)	-0.14	0.21	-0.71	0.98
Laquinimod (5 μM)	-0.25	0.17	-1.49	0.65
AM-251(5 μM)	0.55	0.22	2.46	0.13

inf: infinitesimal

Supplemental Table 6: Time to occlusion batch corrected statistics comparing the effect of drugs versus the DMSO and mestranol control groups, respectively, in Figure 3. These data show the impact of the drugs and mestranol combinations against the reference groups including DMSO and mestranol control**.**

University of Michigan Data			
	HC alone	HC with Thyroid Medication	Thyroid Med Alone
Baseline Characteristics			
Total	47,710	2,125	30,731
Age	25.1 (25.0-25.2)	32.6 (32.2-33.0)	37.7 (37.6-37.8)
BMI	26.0 (25.9-26.1)	25.5 (25.0-25.9)	30.2 (30.1-30.3)
Smoking Status			
Never	79.2%	79.3%	52.0%
Former	8.2%	14.2%	15.0%
Current	5.2%	4.0%	9.1%
Unspecified	7.4%	2.5%	23.2%
Medication			
Thyroid			
Levothyroxine		1850 (87.1%)	27,981 (91.1%)
Reconstituted Thyroid		164 (7.7%)	1,720 (5.6%)
Liothyronine		111 (5.2%)	1,028 (3.4%)
Duration (days)			
		557 (529-586)	847 (834 - 860)
Levothyroxine-eq Dose (mg)			
		0.090 (0.088-0.092)	0.097 (0.096-0.98)
Hormonal contraception			
Estrogen			
Estrogen dose (mg)	0.030 (0.029-0.030)	0.037 (0.031-0.044)	
Estrogen only	18 (<1%)	19 (<1%)	
Progesterone (%)			
1st generation	13984 (29.3%)	762 (36.2%)	
2nd generation	8060 (16.9%)	417 (19.8%)	
3rd generation	21237 (44.5%)	752 (35.7%)	
4th generation	4,409 (9.2%)	173 (8.2%)	
Route (%)			
PO	43979 (92.2%)	1990 (93.7%)	
Vaginal Ring	2,834 (5.94%)	99 (4.6%)	
Patch	895 (1.9%)	34 (1.6%)	
Duration (days)			
	674 (668-681)	557 (528-585)	
Diagnosis			
DVT	131 (41.1%)	8 (32%)	149 (57.8%)
Pulmonary Embolism	79 (24.8%)	12 (48%)	59 (22.9%)
Thrombophlebitis	64 (20.1%)	3 (12%)	17 (6.6%)
Stroke	26 (8.2%)	2 (8%)	6 (2.3%)
Abdominal Thrombosis	12 (3.8%)	-	18 (7.0%)
Arterial Thrombosis	7 (2.2%)	-	9 (3.5%)

Supplemental Table 7: Patient, medication, and diagnosis characteristics.

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