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Combined oral contraceptives-induced coagulopathies
Evaluation of the prothrombotic state using a global coagulation assay

Morimont, Laure

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Combined Oral Contraceptives - Induced Coagulopathies

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Evaluation of the Prothrombotic State Using a Global Coagulation Assay

Submitted by Laure Morimont
for the PhD degree in Biomedical and Pharmaceutical Sciences

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JURY

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Combined Oral Contraceptives-Induced Coagulopathies – Evaluation of the Prothrombotic State Using a Global Coagulation Assay

Twenty-two thousand cases of thrombosis occur each year in Europe following the use of birth control pills containing an oestrogen derivative and a progestin (and qualified as combined oral contraceptives (COCs)). One of the major challenges for healthcare professionals is to identify women at risk. To this end, several guidelines offer support in tailoring contraception, according to the patient's profile. However, these strategies rely on epidemiological data and do not allow for an accurate assessment of the risk of venous thromboembolism (VTE) at the individual level.

Recent data has confirmed activated protein C (APC) resistance, a dysregulation of haemostasis, as an independent VTE risk factor. Although several methods for APC resistance (APCR) detection have been developed, the endogenous thrombin potential (ETP)-based APCR assay has proven to be the most suitable for the assessment of COC-induced changes on the coagulation system. Besides, this test is required by the European Medicines Agency as part of the haematological assessment of the thrombogenic profile of new COC preparations. In addition, as the result is presented as a normalized APC sensitivity ratio (nAPCsr) ranging from 0 to 10, result interpretation is very easy: the higher the nAPCsr, the higher the resistance to APC.

The objective of this thesis is to demonstrate the analytical performances of the ETP-based APCR assay as well as its interest in the routine care setting to aspire to the title of biomarker to characterize the VTE risk of women on contraception.

Although this test was developed more than 20 years ago, it has been underused because the lack of standardization led to an unacceptable variability, hampering the proper evaluation of APCR induced by hormonal therapies in different studies. The first step undeniably consisted in validating the methodology, according to regulatory requirements in terms of analytical performances. To ensure the good reproducibility of the test, we transferred the methodology in two external laboratories to verify that similar results were obtained regardless of the laboratory. Finally, considering its screening potential, the next phase consisted in implementing the ETP-based APCR assay on an automated platform. Indeed, making it available in clinical routine would make the test accessible to prescribers and patients.

In a second step, to illustrate the VTE risk prediction capacities of the nAPCsr, we created exploratory prediction models by combining both the level of APCR (expressed as nAPCsr) for specific COC preparations with their respective VTE relative risk extracted from published epidemiological studies. VTE risk estimated by the models for different birth control pills were similar to those reported in population-based phase IV trials. Actually, these population-based phase IV trials are undertaken following the marketing of a new COC to assess its long-term safety and to identify unusual side effects. In definitive, these models could serve for regulatory purpose since they could permit to avoid waiting almost a decade before positioning on the potential risk of VTE of a particular COC (currently set only at the end of population-based phase IV trials). It may also save time and money for the pharmaceutical companies and the regulators and may facilitate the uptake of innovative compounds for the clinicians by providing reassuring data on the risk of VTE with hormonal therapies.

Lastly, in 2021, a new COC containing 15 mg of estetrol and 3 mg of drospirenone was marketed. The haemostasis profile investigated during the phase II clinical trial revealed a moderate effect of this preparation on the nAPCsr. In agreement with the pharmaceutical company which produces it, we further explored data originating from the ETP-based APCR assay. Thanks to these investigations, we could confirm the lower impact of this new COC on the global coagulation process compared to the most commonly used birth control pills (containing ethinylestradiol as oestrogen derivative).

As a perspective, we are convinced that the nAPCsr has the potential to become a safety biomarker to assess the VTE risk in women on COCs and a surrogate endpoint allowing an in-silico evaluation of the VTE risk profile of new COC preparations before population-based phase IV trials are completed. Starting the procedure of biomarker qualification by the European Medicine Agency will represent the next step. Future research should also focus on the extended use of the nAPCsr. We have already demonstrated that it could be useful in other hormone-induced coagulopathies, such as during pregnancy and in the postpartum period or due to the intake of menopausal replacement therapies or tamoxifen therapy in breast cancer patients. Last but not least, as mentioned in the last ISTH SSC communication, the scope of this test goes beyond hormone-induced prothrombotic states and might be relevant in other pathologies such as haematological cancer and antiphospholipid syndrome.

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Les Coagulopathies Induites par les Contraceptifs Oraux Combinés – Évaluation de l'État Prothrombotique à l'Aide d'un Test Global de la Coagulation

22 000 cas de thromboses surviennent chaque année en Europe suite à l'utilisation de contraceptifs oraux combinés (« *combined oral contraceptives* », COCs). L'un des principaux challenges pour les professionnels de la santé est d'identifier ces femmes à risque. À cette fin, plusieurs lignes directrices offrent un soutien pour adapter la contraception en fonction du profil de la patiente. Cependant, ces stratégies reposent sur des données épidémiologiques et ne permettent pas une évaluation précise du risque de thromboembolie veineuse («*venous thromboembolism* », VTE).

Des données récentes ont confirmé qu'une résistance à la protéine C activée (« *activated protein C* », APC), considérée comme un dysfonctionnement au niveau de l'hémostase, représente un facteur de risque indépendant de VTE. Bien que plusieurs tests aient été développés, c'est le test de résistance à l'APC basé sur le potentiel de thrombine endogène (« *endogenous thrombin potential* », ETP) qui s'est montré le plus sensible pour évaluer les changements induits par les COCs sur le système de la coagulation. Ce test est d'ailleurs un requis règlementaire par l'Agence Européenne des Médicaments dans le cadre de l'évaluation du profil thrombogène des COCs en étude clinique. De plus, les résultats de ce test peuvent être exprimés en un ratio allant d'une échelle de 0 à 10, appelé « *normalized activated protein C sensitivity ratio* (nAPCsr) », ce qui offre une interprétation aisée des résultats : au plus le nAPCsr est élevé, au plus la résistance à l'APC est importante.

L'objectif de cette thèse est de démontrer les performances analytiques du test de résistance à l'APC basé sur l'ETP ainsi que son intérêt en routine clinique pour prétendre au titre de biomarqueur afin de caractériser le risque de VTE chez les femmes sous contraception.

Bien que ce test ait été développé il y a plus de 20 ans, il a longtemps été mis de côté, car le manque de standardisation a conduit à une variabilité inacceptable, empêchant la bonne évaluation de la résistance à l'APC induite par les thérapies hormonales. La première étape a indéniablement consisté à valider la méthodologie, selon les exigences règlementaires en termes de performances analytiques. Afin d'assurer la bonne reproductibilité du test, nous avons transféré la méthodologie dans deux laboratoires externes pour vérifier que des résultats similaires étaient

obtenus, quel que soit le laboratoire. Enfin, compte tenu de son potentiel de dépistage, la phase suivante a consisté à implémenter le test de résistance à l'APC basé sur l'ETP sur une plateforme automatisée. En effet, sa mise à disposition en routine clinique rendrait le test accessible aux prescripteurs et aux patients.

Dans un deuxième temps, pour illustrer les capacités de prédiction du risque de VTE du nAPCsr, nous avons créé des modèles de prédiction exploratoires en combinant à la fois le niveau de résistance à l'APC (exprimé en nAPCsr) de différents COCs avec leur risque relatif de VTE extrait d'études épidémiologiques. Le risque de VTE a ensuite été estimé, sur base de ces modèles, pour deux pilules contraceptives bien distinctes. Le risque était alors similaire à celui rapporté dans les études dites de phase IV ou de pharmacovigilance. Ces études de phase IV sont entreprises après la mise sur le marché d'un nouveau médicament afin d'évaluer sa sécurité à long terme et d'identifier les effets secondaires inhabituels. *In fine,* ces modèles pourraient servir à des fins règlementaires puisqu'ils pourraient permettre d'éviter d'attendre près d'une décennie avant de se positionner sur le risque potentiel de VTE d'un COC particulier (actuellement déterminé par les études de pharmacovigilance). Ils pourraient représenter un gain de temps pour les entreprises pharmaceutiques et les organismes de règlementation et faciliter l'utilisation de composés innovants par les cliniciens en fournissant des données rassurantes sur le risque de VTE associé aux thérapies hormonales.

Enfin, en 2021, un nouveau COC contenant 15 mg d'estétrol et 3 mg de drospirénone a été commercialisé. Le profil d'hémostase étudié lors de l'étude clinique de phase II a révélé un effet modéré de cette préparation sur le nAPCsr. En accord avec la société pharmaceutique qui le produit, nous avons exploré plus profondément les données provenant du test de résistance à l'APC basé sur l'ETP. Grâce à ces investigations, nous avons pu confirmer le moindre impact de ce nouveau COC sur le processus global de coagulation par rapport aux pilules contraceptives les plus couramment utilisées (contenant de l'éthinylestradiol comme dérivé estrogénique).

En perspective, nous sommes convaincus que le nAPCsr pourrait devenir un biomarqueur de sécurité pour évaluer le risque de VTE chez les femmes sous COC et un biomarqueur de substitution permettant une évaluation rapide du profil de risque de VTE des nouveaux COCs avant la fin des études de pharmacovigilance. La prochaine étape consistera à démarrer la procédure de qualification de biomarqueur auprès de l'Agence Européenne du Médicament. Les recherches futures devraient également se concentrer sur l'utilisation étendue du nAPCsr. Nous avons déjà démontré qu'il pourrait être utile dans d'autres coagulopathies d'origine hormonales. Par exemple, durant la grossesse et la période de postpartum ou suite à la prise d'un traitement hormonal de substitution chez les femmes ménopausées, ou encore, de tamoxifène chez les personnes souffrant d'un cancer du sein

hormono-dépendant. Enfin, comme mentionné dans la dernière communication de la SSC de l'ISTH, le champ d'application de ce test va au-delà des états prothrombotiques induits par les hormones et pourrait être pertinent dans d'autres pathologies telles que les hémopathies malignes et le syndrome des antiphospholipides.

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Avant d'entamer votre lecture, qui sera probablement longue et fastidieuse, mais au terme de laquelle, du moins je l'espère, le risque de thrombose associé aux contraceptifs oraux combinés n'aura plus aucun secret pour vous, je souhaiterais m'adresser aux personnes ayant contribuées de près ou de loin à l'aboutissement de ce projet.

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alors je vais être brève. Le principe de l'amitié, c'est les 3C. Complicité, Confiance, Conneries, tout simplement ce que je partage avec vous.

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Comme l'a dit un jour Bill Clinton, *« Nous ne pouvons construire notre propre avenir sans aider les autres à construire le leur »* et je vous remercie du fond du cœur d'avoir participé à la construction du mien.

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List of abbreviations

Ala	Alanine
ANOVA	Analysis of variance
APC	Activated protein C
APCR	Activated protein C resistance
APCsr	Activated protein C sensitivity ratio
aPTT	Activated partial thromboplastin time
Arg	Arginine
Asp	Aspartic acid
AT	Antithrombin
BMI	Body mass index
CAV-1	Caveolin-1
CAT	Calibrated Automated Thrombogram
CD	Critical difference
CEE	Conjugated equine oestrogen
CHC	Combined hormonal contraceptive
CHMP	Committee for Medicinal Products for Human Use
CHU	Centre Hospitalier Universitaire
CLSI	Clinical and Laboratory Standards Institute
CMA	Chlormadinone acetate
CPA	Cyproterone acetate
CPMP	Committee for Proprietary Medicinal Products
COC	Combined oral contraceptive
CV	Coefficient of variation
DNA	Deoxyribonucleic acid
DNG	Dienogest
DOAC	Direct oral anticoagulant
DRSP	Drospirenone
DSG	Desogestrel
EE	Ethinylestradiol
EMA/EMEA	European Medicines Agency
EPCR	Endothelial protein C receptor
ER	Oestrogen receptor
ERE	Oestrogen response element
ERK	Extracellular signal-regulated kinase
ETP	Endogenous thrombin potential
EWP	Efficacy Working Party
E1	Oestrone

E2(V)	Oestradiol (valerate)
E3	Oestriol
E4	Estetrol
FDA	Food and Drug Administration
FSH	Follicle stimulating hormone
FVIII(a)	(Activated) Factor VIII
FVIIIi	Inactivated Factor VIII
FIX(a)	(Activated) Factor IX
FV(a)	(Activated) Factor V
FVi	Inactivated factor V
FVL	Factor V Leiden
FX(a)	(Activated) Factor X
Gai	G-coupled protein ai
GSD	Gestodene
GSM	Genitourinary syndrome of menopause
HPP	Healthy pooled plasma
ICH	International council for harmonization
Ile	Isoleucine
INAS-SCORE	International Active Surveillance study "Safety of Contraceptives: Role of Oestrogens"
IQC	Internal quality control
ISTH	International Society on Thrombosis and Haemostasis
IVD	In-vitro diagnostic
LNG	Levonorgestrel
LT	Lag time
MAE	Maximal allowable error
MAPK	Mitogen-activated protein kinase
MISS	Membrane-initiated steroid signalling
MRT	Menopausal replacement therapy
mVRI	Mean velocity rate index
NAB-X	Namur Biobank-eXchange
nAPCsr	Normalized activated protein C sensitivity ratio
NAS	New active substance
NEST	Native oestrogen with specific action in tissues
NETA	Norethindrone/norethisterone acetate
NGM	Norgestimate
NIH	National Institutes of Health
NO	Nitric oxide
NOMAC	Nomegestrol acetate

NPP	Normal pooled plasma
OC	Oral contraceptive
PC	Protein C
PH	Peak Height
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PLs	Phospholipids
PPP	Platelet poor plasma
PRAC	Pharmacovigilance Risk Assessment Committee
PRO-E2	Prospective Controlled Cohort Study on the Safety of a Monophasic Oral Contraceptive Containing Nomegestrol Acetate (2.5mg) and 17β-oestradiol (1.5mg)
PS	Protein S
PT	Prothrombin
QC	Quality control
RL	Rejection limit
RR	Relative risk
RVV	Russel viper venom
SD	Standard deviation
SERM	Selective oestrogen receptor modulator
SHBG	Sex hormone binding globulin
SI	Synergy index
SMPC	Summary of products characteristics
SSC	Scientific and Standardization Committee
TAT	Thrombin-antithrombin
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TGA	Thrombin generation assay
TGT	Thrombin generation test
TM	Thrombomodulin
Trh	Threonine
Trp	Tryptophan
TS	STG® ThromboScreen - TM
TTP	Time to peak
TVUS	Transvaginal ultrasound
UCL	Université Catholique de Louvain
US	United States
Val	Valine
VMS	Vasomotor symptoms

VTE	Venous thromboembolism
VWF	Von Willebrand Factor

“This manuscript is a compilation of scientific articles written during my cursus, but these have been updated or adjusted to avoid redundancy and ease the reading. On that note, I wish insightful reading”

INTRODUCTION

Review in Frontiers in Endocrinology. 2021; 12

COMBINED ORAL CONTRACEPTIVES AND VENOUS THROMBOEMBOLISM: REVIEW AND PERSPECTIVE TO MITIGATE THE RISK

Laure Morimont, Hélène Haguet, Jean-Michel Dogné, Ulysse Gaspard, Jonathan Douxfils

SUMMARY

Many factors must be considered and discussed with women when initiating a contraceptive method and the risk of venous thromboembolism (VTE) is one of them. In this review, we discuss the numerous strategies that have been implemented to reduce the thrombotic risk associated with combined oral contraceptives (COCs) from their arrival on the market until today. Evidence suggesting that COCs were associated with an increased risk of VTE appeared rapidly after their marketing. Identified as the main contributor of this risk, the dosage of the oestrogen, i.e., ethinylestradiol (EE), was significantly reduced. New progestins were also synthesized (e.g., desogestrel or gestodene) but their weak androgenic activity did not permit them to counterbalance the effect of EE as did the initial progestins such as levonorgestrel. Numerous studies assessed the impact of oestroprogestative combinations on haemostasis and demonstrated that women under COC suffered from resistance towards activated protein C (APC). Subsequently, the European Medicines Agency updated its guidelines on clinical investigation of steroid contraceptives in which they recommended assessing this biological marker. In 2009, oestradiol-containing COCs were marketed and the use of this natural form of oestrogen was found to exert a weaker effect on the synthesis of hepatic proteins compared to EE. In 2021, a novel COC based on a native oestrogen, i.e., estetrol, was introduced on the market. Associated with drospirenone, this preparation demonstrated minor effects on coagulation proteins as compared with other drospirenone-containing COCs. At the present time, the standard of care when starting contraception, consists of identifying the presence of hereditary thrombophilia solely based on familial history of VTE. This strategy has, however, been reported as poorly predictive of hereditary thrombophilia. One rationale and affordable perspective which has already been considered in the past could be the implementation of a baseline screening of the prothrombotic state to provide healthcare professionals with objective data to support the prescription of the more appropriate contraceptive method. While this strategy was judged too expensive due to limited laboratory solutions, the endogenous thrombin potential-based APC resistance assay could now represent an interesting alternative.

INTRODUCTION

When oral contraceptives became generally available during the early 1960s, their use rapidly increased and in 2019, it was estimated that over 150 million women were using the pill worldwide (United Nations, 2019). With such many subjects on this medication, and with a majority using combined oestrogen-progestin products, even a small increase in risk of serious side effects affects the lives of many women. Moreover, as contraceptive therapies are administered to healthy young women with the aim to prevent unwanted pregnancies, the occurrence of side effects should be as low as possible and risk minimization strategies should be implemented accordingly.

1960 – THE BEGINNING OF COMBINED ORAL CONTRACEPTIVES ERA

Evidence suggesting that oral contraceptives were associated with an increased risk of venous thromboembolism (VTE) events appeared rapidly after they were marketed **(Figure 0.1).** The first case of VTE was reported in 1961 in a 40-year-old woman who had been given Enovid®, an association of 150µg of mestranol and 10mg of norethynodrel for the control of endometriosis (Jordan et al., 1961; Stadel, 1981). This association of an oestrogen with a progestin marked the beginning of the combined hormonal contraceptives (CHCs) era which were initially only given by the oral route. In the subsequent years, it was reported that the occurrence of VTE was higher with combined oral contraceptives (COCs) containing more than 50µg of oestrogen, either mestranol or ethinylestradiol (EE), compared to preparations containing a lower dosage (Inman et al., 1970). Following the publication of these reports, the British Committee on Safety of Drugs, one of the leading instances for drug safety at that period, issued, in 1969, a statement indicating that the dose of oestrogen contained in oral contraceptives was positively associated with an increased risk of VTE (Vessey et al., 1973). The dosage of oestrogen was then reduced from 75µg, or more, to 50µg and afterwards to 30 and 20µg. At the present time, some COCs even contain 10µg of EE (e.g., Lo-Loestrin® Fe). Comparisons of COCs containing 50 and 30µg of EE on blood coagulation, fibrinolysis and platelets confirmed the oestrogen dose-dependent effect (Meade et al., 1977). Enhanced platelet activity, increased levels of factors II, VII, VIII, IX and X, fibrinogen and soluble fibrin and decreased levels of antithrombin (AT) and vessel wall fibrinolytic activator were observed with both preparations, but they were less pronounced with 30µg COC preparations **(Figure 0.2)** (Bonnar, 1987; Meade et al., 1977; Stadel, 1981; Wessler et al., 1976).

The progestin compound found in oral contraceptives also changed over time with several pharmacomodulations aiming at providing different oestrogenic, androgenic, glucocorticoid or mineralocorticoid profiles (Wiegratz et al., 2006).

Indeed, endogenous progesterone, synthesized in the ovarian corpus luteum, possesses antiestrogenic, antiandrogenic and anti-mineralocorticoid activities. Thus, synthetic progestogens, i.e., progestins, used for contraception also mimicked some of these properties (Blanco-Molina et al., 2012).

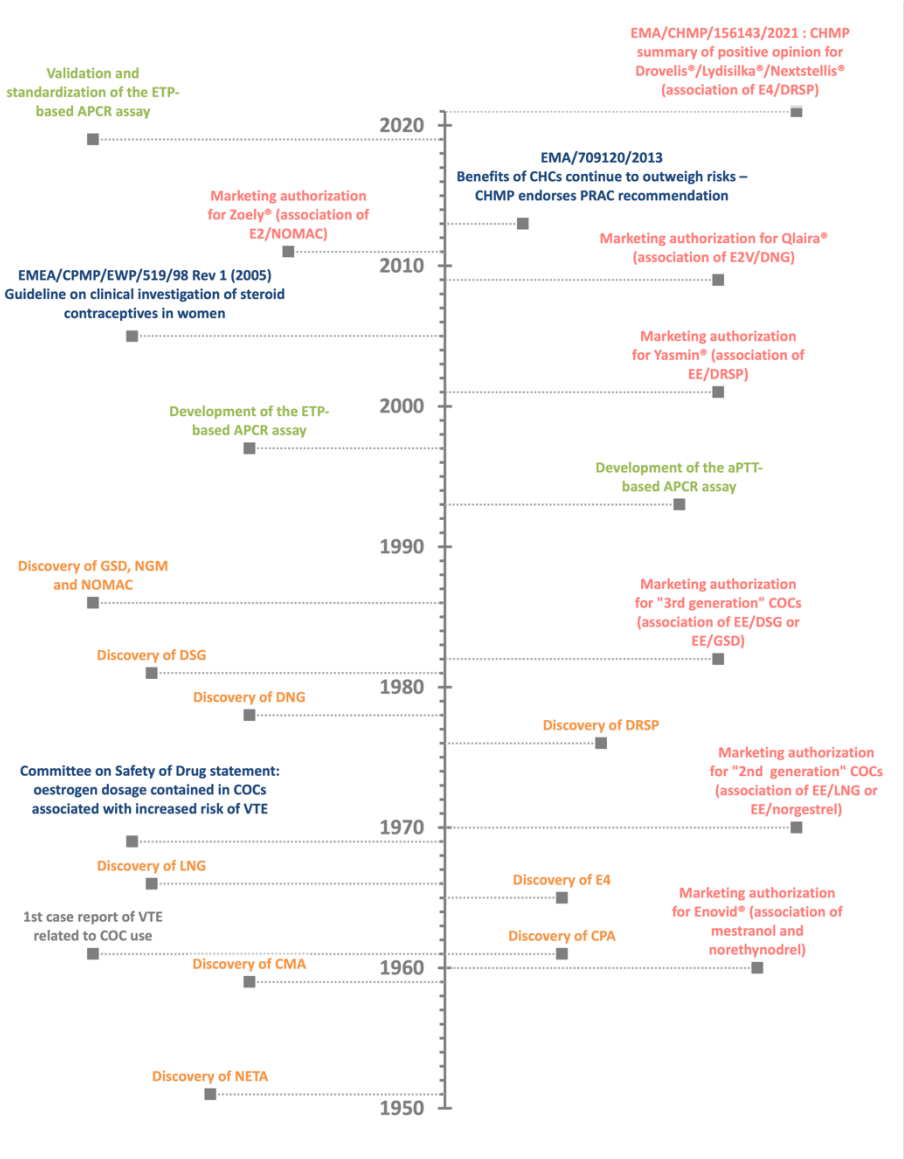


Figure 0.1 | The History of COC and the related risk of VTE.
Discovery of oestrogen and progestin compound (in orange) – marketing authorization for COCs (in red) – authorities' statement (in blue) – assay development (in green).

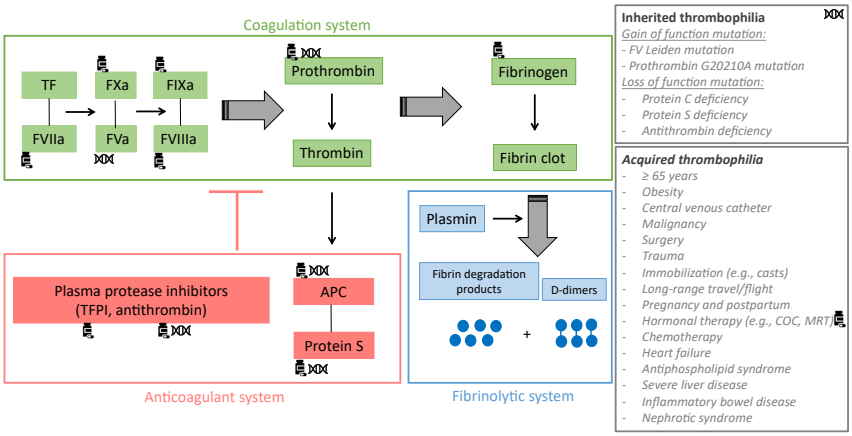


Figure 0.2 | Coagulation, fibrinolytic and anticoagulant systems.
The green rectangle refers to the coagulation system, the blue rectangle refers to the fibrinolytic system and the red rectangle refers to the anticoagulant system. Tissue factor (TF) plays an important role in the initiation of the coagulation process. Combined with activated factor VII (FVIIa), the complex activates factor IX (FIX) and factor X (FX). Subsequently, activated FIX (FIXa) and FX (FXa) form two complexes with activated FVIII(FVIIIa) and activated FV(FVa) respectively, leading to the conversion of prothrombin into thrombin. Once formed, thrombin cleaves fibrinogen to form the fibrin clot which is then degraded by the fibrinolytic system whose main effector is plasmin. This releases fibrin degradation products and D-Dimers. Thrombin also activates the protein C system to down regulate its own production. Indeed, once activated by thrombin, protein C forms a complex with protein S to inactivate FVa and FVIIIa, the two main cofactors of the intrinsic (FIXa-FVIIIa) and the prothrombinase (FXa-FVa) complexes. The generation of thrombin is also regulated by other protease inhibitors like antithrombin (AT) and tissue factor pathway inhibitor (TFPI). Inherited and acquired thrombophilia can disrupt the coagulation, the fibrinolytic or the anticoagulant system leading to an increased risk of venous thromboembolism. Coagulation factors impacted by inherited thrombophilia are marked with the symbol ⋈ and those impacted by the intake of COC are marked with the symbol .

Overall, these compounds are characterized by a 4-ring steroid skeleton and are classified based on their structure **(Table 0.1).** The first progestins, synthesized in 1951, were norethynodrel and norethisterone acetate (also known as norethindrone acetate). They are characterized by a 19-nortestosterone structure and are regarded as estranes (carbon-18). Shortly afterwards, in 1959, 17-hydroxyprogesterone derivatives categorized as pregnanes (carbon-21) were also synthesized. These included chlormadinone acetate and cyproterone acetate. In 1966, other 19-nortestosterone derivatives were discovered with norgestrel and levonorgestrel but differed from norethynodrel and norethisterone acetate as they possess a 17-carbon structure. They are better known as gonanes. During the 1980s, three new progestins, derived from levonorgestrel, were developed that are desogestrel, gestodene and norgestimate. Drospirenone, dienogest and nomegestrol acetate are, for their part, considered to be the new progestin

Table 0.1 | Progestins - Discovery, chemical structure, androgenic activity, associated oestrogen compound in COC and classification under generation.

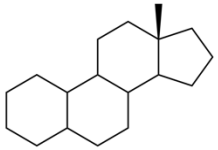
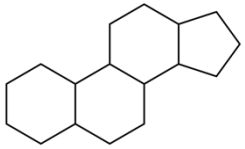
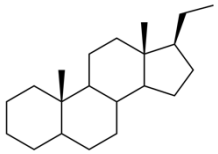
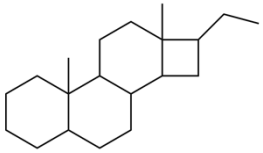
Progestins	*Short name*	*Discovery date*	*Chemical structure*	*Androgenic activity*	*Anti-androgenic activity*	*Associated oestrogen compound in COC*	*Classification under generation*
Norethisterone acetate	NETA	1951	Estrane (18-carbon structure) 	+	-	EE ≥ 50µg	1st generation COC
Norethynodrel	/	1957		+	-		
Lynestrenol	/	1961		+	-		
Levonorgestrel	LNG	1966	Gonane (17-carbon structure) 	++	-	EE 20 µg/ 30 µg	2nd generation COC
Norgestrel	/	1966		++	-		
Desogestrel	DSG	1981		+	-	EE 20µg/ 30 µg	3rd generation COC
Gestodene	GSD	1986		+	-		
Norgestimate	NGM	1986		+	-		

Table 0.1 | (continued) Progestins - Discovery, chemical structure, androgenic activity, associated oestrogen compound in COC and classification under generation.

Progestins	*Short name*	*Discovery date*	*Chemical structure*	*Androgenic activity*	*Anti-androgenic activity*	*Associated oestrogen compound in COC*	*Classification under generation*
Chlormadinone acetate	CMA	1959	Pregnane (21-carbon structure) 	-	++	EE 30µg	Unclassified/other
Cyproterone acetate	CPA	1961		-	++	EE 35µg	
Drospirenone	DRSP	1976	Spironolactone derivative	-	+	EE 20µg/ 30µg	
Dienogest	DNG	1978	Gonane (17-carbon structure)	-	+	EE 30µg E2V 1-3mg	
Nomegestrol acetate	NOMAC	1975	Norpregnane (20-carbon structure) 	-	+	Micronized E2 1.5 mg	

They were designed to bind more specifically to the progesterone receptors and to a lesser extent to the other steroid receptors to reduce undesirable effects. Regarding their structure, dienogest is a gonane derivative, nomegestrol acetate is a norpregnane (carbon-20) derivative and drospirenone is unique in its category and derives from spironolactone **(Table 0.1)**(Kuhl, 2005; Lawrie et al., 2011; Regidor, 2018; Sitruk-Ware, 2006, 2008).

Combined oral contraceptives were usually classified into three generations, related to their arrival on the market. First generation contained high doses of EE (50µg or more) associated with norethynodrel and with norethisterone acetate. They are no longer used in COC preparations. Second and third generations contain a lower dose of EE (20 or 30µg). The associated progestin is levonorgestrel in 2nd generation COCs and desogestrel, gestodene or norgestimate in 3rd generation COCs. Actually, this nomenclature was introduced by the pharmaceutical companies with the aim of boosting sales since the idea of a "new" generation suggests improvements and better efficacy and/or safety profile, the two latter being not supported by epidemiological data (Creinin et al., 2020). This misleading classification led to inconsistencies like norgestimate-contained COC, categorized as a 3rd generation, which is in fact a prodrug of levonorgestrel and its 3-oxime metabolite, renamed norelgestromin by the pharmaceutical company (Kuhnz et al., 1994). Although it is tempting to turn then to a chemical classification, this is not the Holy Grail since among compounds with nearly similar chemical structure, e.g. levonorgestrel and gestodene, the pharmacodynamic action when binding to the different steroid receptors may differ (Kuhl, 2011). Therefore, a pharmacodynamic classification should be preferred considering the activities of the progestin when associated with an oestrogen, the potency of the latter being a determinant of the total estrogenicity of the association.

1980 - THIRD GENERATION AND NEW ORAL CONTRACEPTIVES CONTROVERSY

Going back into the 80s, the main reason that led to the development of new progestins was related to the side effects induced by the combination of EE with levonorgestrel or norethisterone acetate, such as acne, hirsutism and weight gain (LeBlanc et al., 1999). The decreased androgenic activity of desogestrel, gestodene and norgestimate has certainly enabled to reduce these adverse effects. However, an increased risk of VTE was observed compared to levonorgestrel as reported by 4 independent epidemiological studies in 1995-1996 (Bloemenkamp et al., 1995; Jick et al., 1995; Spitzer et al., 1996; World Health Organization Collaborative Study of Cardiovascular Disease and Steroid Hormone Contraception, 1995). Although these results have been debated due to the possible impact of confounding factors and bias, such as healthy user bias, introduction bias, duration of oral contraceptive use,

COC switching, prescribing bias, diagnosis suspicion and referral bias or the source of funding, the increased risk with these progestins has been subsequently confirmed by other investigations (de Bastos et al., 2014; Farmer et al., 1997; Heinemann, 1999; Lewis et al., 1996; Spitzer, 1999; Suissa et al., 1997). As earlier studies suggested that the increased risk of VTE associated with COCs was only oestrogen dose-dependent, the most plausible explanations at that time were these biases. Nevertheless, the meta-analysis of *Kemmeren et al.* published in 2001, revealed that even after stratifying for various factors like first-time users; age (i.e. younger and older than 25 years); duration of use (i.e. less or more than 1 year); confirmation status of VTE cases and source of funding (i.e. industry or non-industry-sponsored study); the risk remained more elevated with the so-called 3rd generation COCs compared to the 2nd generation COCs (Kemmeren et al., 2001).

This phenomenon was also observed with COCs containing EE associated with drospirenone and cyproterone acetate. Unlike levonorgestrel, desogestrel, gestodene and norgestimate, the latter two progestins are completely devoid of any androgenic or glucocorticoid effects (Sitruk-Ware, 2005). Cyproterone acetate possesses even the highest antiandrogenic activity which makes this molecule the ideal candidate to treat severe acne and hirsutism in women (Kuhl, 2011). Drospirenone, on the other hand, differs from the other progestins by its chemical structure derived from spironolactone conferring an antimineralocorticoid activity. This allows the offset of the fluid retention induced by oestrogens and prevent weight gain during COC therapy (Foidart, 2005). The development of these two progestins with antiandrogenic and antimineralocorticoid properties provided us with compounds closer to progesterone permitting the reduction of the above-mentioned adverse effects but on the flip side of the coin, they clearly led to an increased risk of VTE when associated with EE (Rosendaal et al., 2003; van Vliet et al., 2004).

As evidence demonstrated that COCs with the same oestrogen dose but different progestins were associated with differential VTE risk, it was suggested that the progestin compound might play a role in thrombosis development. However, as progestin only contraceptives do not interfere with coagulation protein synthesis, the difference in VTE risk specific to each COC could only be attributed to a distinctive modulation of the procoagulant effect of EE, exerted by the progestins (Fruzzetti et al., 2018; Tepper et al., 2016). This modulation is actually related to the activity of the progestin compound on hormonal receptors and especially on androgen receptors (Kuhl, 1996). Levonorgestrel, characterized by a strong androgenic activity, balances out to a certain degree the oestrogen-dependent alteration in haemostasis and hepatic protein synthesis. Consequently, levonorgestrel further offsets the procoagulant effect induced by EE compared to desogestrel, gestodene,

norgestimate, drospirenone and cyproterone acetate, which have a weaker androgenic or even an antiandrogenic activity (Kuhl, 2011; Lawrie et al., 2011; Regidor, 2018). An interaction between androgen receptors and oestrogens responsive elements could be a hypothesis to explain this phenomenon (Peters et al., 2009). It could prevent the activation of target genes coding for hepatic proteins such as coagulation factors, thus modulating the effect of EE (Peters et al., 2009). Ultimately, the estrogenicity of a COC is the sum of both the oestrogen and the progestin contribution and excessive estrogenicity was reported to increase the risk of VTE **(Figure 0.3)** (Odlind et al., 2002).

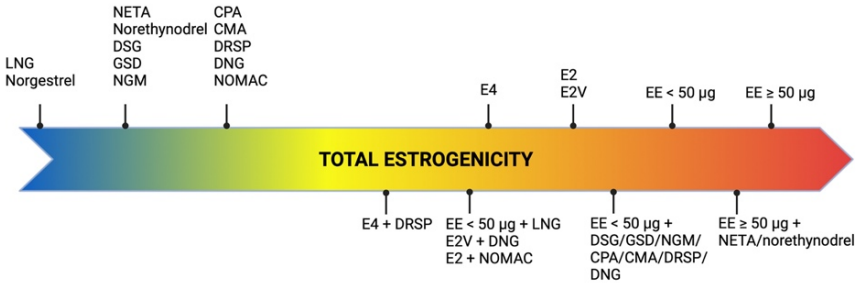


Figure 0.3 | Concept of estrogenicity with COCs.

The biomarker that best reflects the estrogenicity is the sex hormone-binding globulin (SHBG), a carrier protein for oestrogen and testosterone, produced by the liver, and whose synthesis is highly oestrogen sensitive (Odlind et al., 2002). The oral intake of EE alone leads to a significant dose-dependent increase in SHBG whereas progestins induce a decrease of SHBG, the extent being dependent of its androgenic activity (Odlind et al., 2002). Thus, progestins with a potent androgenic activity cause a more pronounced reduction in SHBG levels than less androgenic or antiandrogenic ones. Consequently, the so-called 3rd generation COCs and those containing EE combined with drospirenone or cyproterone acetate induce a drastic increase in SHBG levels compared to the so-called 2nd generation COCs (Odlind et al., 2002; Van Rooijen et al., 2004; van Vliet et al., 2005).

As a relationship has been observed between SHBG levels and the increased risk of VTE associated with COC use, the assessment of this liver protein represents an important biomarker to consider during the development of steroid contraceptives (European Medicines Agency, 2005; Raps et al., 2012). However, SHBG is not a coagulation protein and other biological variables better reflecting the impact of COCs on haemostasis would seem more appropriate to reflect the potential induced prothrombotic switch (Stegeman et al., 2012).

1990 - ACQUIRED ACTIVATED PROTEIN C RESISTANCE WITH ORAL CONTRACEPTIVES: A KEY FACTOR IN VENOUS THROMBOEMBOLISM RISK

In the 1990s, it was estimated that VTE affected 1 per 1,000 people annually, and that family history of thrombosis was often found in these patients (Dahlbäck, 1994). However, the main inherited thrombophilia investigated at that time such as deficiency of antithrombin, protein C and protein S accounted for only 5- to 10% of the cases (Dahlbäck, 1994). This suggested that other genetic defects predisposed to the development of VTE and had yet to be identified. In 1993, *Dahlbäck et al.* started to investigate the protein C - protein S anticoagulant pathway (Dahlbäck et al., 1993). They developed an activated partial thromboplastin time (aPTT)-based method to determine the sensitivity of a patient's plasma towards exogenous activated protein C (APC). The aPTT assay is based on the principle that in citrated plasma, the addition of phospholipids (PLs), activator of factor XII (e.g., silica) and calcium chloride allow for the formation of a stable clot. The time from activation to formation of a stable clot is recorded in seconds, and represents the aPTT (Roshal et al., 2019). After the addition of APC, they noted that plasma from patients with thrombosis and family history of thrombosis had a shorter prolongation of the aPTT clotting time compared with plasma from healthy individuals (Dahlbäck et al., 1993). Therefore, as APC is expected to delay the aPTT in normal plasma, due to its ability to inactivate factor Va and factor VIIIa, the observed phenomenon was defined as APC resistance (APCR). To ease the expression of results, an APC sensitivity ratio, defined as the aPTT(+APC) divided by the aPTT(-APC), was calculated and is depicted in **Equation 1**:

Equation 1 :

$$\text{APCsr (aPTT)} = \frac{sample\ aPTT\ (+APC)}{sample\ aPTT\ (-APC)}$$

The more resistant a sample is to APC, the lower the numerator (+APC condition) compared to the denominator (-APC condition) and therefore the closer the ratio is to zero (Griffin et al., 1993). These findings were confirmed by others (Koster et al., 1993; Svensson et al., 1994) and the phenotype of APCR was later associated with a mutation in the coding region of factor V, better known as Factor V Leiden (FVL) mutation (Bertina et al., 1994). This mutation abolishes one of the APC-cleavage sites on FVa which leads to a slowdown inactivation of FVa. This further prevents FVa from being converted into a functional APC cofactor needed for FVIIIa inactivation and, as a consequence, the inactivation of both FVa and FVIIIa are delayed (Segers et al., 2009). The discovery of this genetic mutation indirectly permitted to better understand the aetiology of COC-induced VTE events. Indeed, many patients were found to have some resistance to APC without carrying a FVL mutation, suggesting either the existence of other genetic mutations or the existence of acquired factors capable of interfering with the inhibitory activity of APC (Bertina et al., 1994). In the

study of *Koster et al*., healthy men had a more pronounced anticoagulant response to APC than women, suggesting a possible influence of female sexual hormones (Koster et al., 1993). It was then assumed that a poor response to APC could explain, at least in part, the procoagulant state observed in users of oral contraceptives. This was confirmed in 1995 by *Henkens et al.* and *Olivieri et al.* who demonstrated that COC therapy could induce acquired APCR independently from genetic mutation of the FVL (Henkens et al., 1995; Olivieri et al., 1995). Although these results were tremendous and important to support the observed increased risk of VTE with COCs and conditions leading to APCR, other tests which account for the entire coagulation process were developed.

In 1997, *Nicolaes et al.* reported on a new method based on the continuous measurement of thrombin generation over time, in the presence and absence of exogenous APC (Nicolaes et al., 1997). Thrombin generation assay (TGA) is based on the potential of plasma to generate thrombin over time, after activation of coagulation by addition of PLs, tissue factor (TF) and calcium. The resulting thrombin generation curve reflects all the pro- and anticoagulant reactions that regulate both thrombin formation and inhibition. In contrast to the aPTT assay, which only assesses the initiation phase of the coagulation, TGA is a global assay investigating the initiation, the propagation, and the termination phase of the coagulation. The addition of APC induces a lowering of thrombin generation which is quantitated by the endogenous thrombin potential (ETP), corresponding to the area under the thrombin generation curve **(Figure 0.4).** This test was therefore referred to as the ETP-based APCR assay and results were expressed as normalized APC sensitivity ratio (nAPCsr). This unit corresponds to the ratio of the ETP measured in presence and absence of APC divided by the same ratio determined in a reference plasma **(Equation 2).**

Equation 2 :

$$\text{nAPCsr} = \frac{Sample\ plasma\ ETP\ (+APC) \Big/ Sample\ \ plasma\ ETP\ (-APC)}{Reference\ plasma\ \ ETP\ (+APC) \Big/ Reference\ plasma\ ETP\ (-APC)}$$

The obtained ratio stands between 0 and 10 and conversely to the aPTT-based assay, the higher the nAPCsr, the more resistant the patient is to APC. A comparison between both tests is shown in **Table 0.2**.

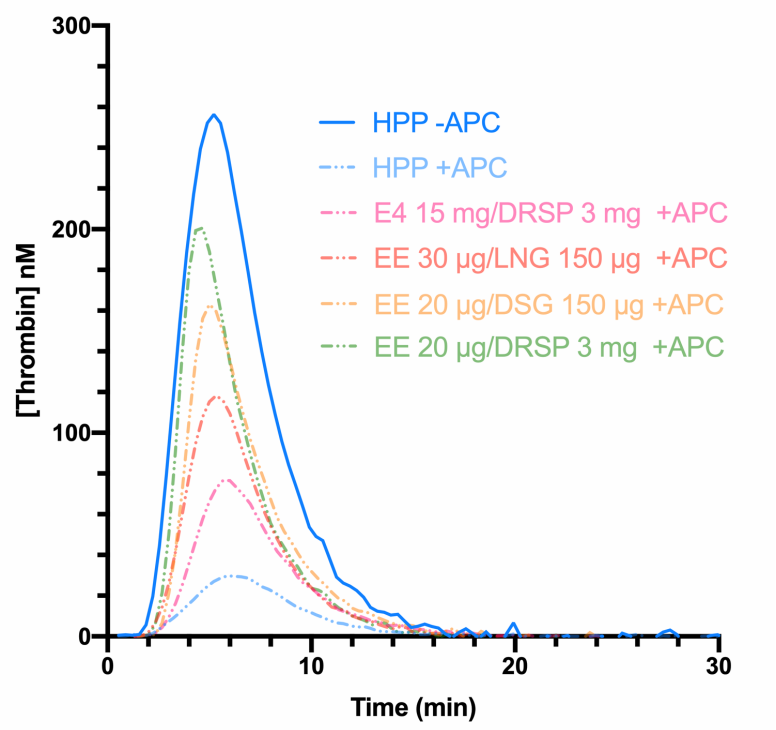


Figure 0.4 | Thrombin generation curves in absence (continuous line) and in presence of APC (dotted lines) of a healthy pooled plasma (blue) and women using COC containing either EE with drospirenone (green), EE with desogestrel (yellow), EE with levonorgestrel (red), or E4 with drospirenone (pink).
The area under the curve represents the ETP- parameter. In presence of APC, the ETP is higher with the use of COCs (e.g., drospirenone or desogestrel) compared to the healthy pooled plasma (composed of men and women not using hormonal contraception), leading to some resistance towards the APC.

Depending on the test used, i.e., aPTT-based or ETP-based APCR assay, the differences observed between nonusers and COC users were not the same. The ETP-based APCR assay revealed to be more sensitive to the COC impact on haemostasis and significant differences were observed between COC preparations (e.g., levonorgestrel-containing products versus desogestrel- or gestodene-containing products) **(Figure 0.4)** (Curvers et al., 1999; Kuhl et al., 1982). Indeed, these two assays are not sensitive to the same factors: the aPTT-based assay is more sensitive towards levels of prothrombin and FVIII while the ETP-based assay is most influenced by free TFPI and free PS levels (de Visser et al., 2005). As these latter factors are much more impacted by COCs than the two formers, it may in part explain the inconsistent results between these two functional APCR assays (Castoldi et al., 2010; Tchaikovski et al., 2006).

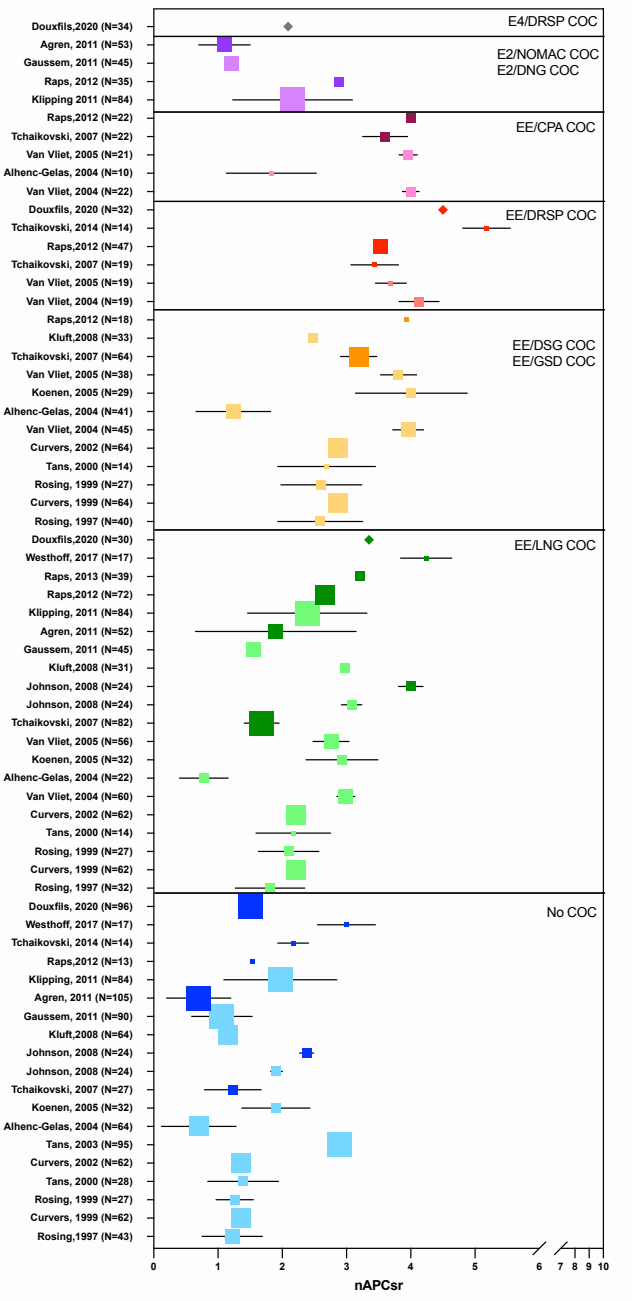
Table 0.2 | Assessment of acquired APCR.

Assay	*aPTT-based APCR assay*	*ETP-based APCR assay*
Development year	1993	1997
Principle	Relies on the ability of APC to prolong the aPTT, via its anticoagulant properties	Relies on the ability of APC to reduce thrombin generation, via its anticoagulant properties.
Trigger	Via intrinsic pathway	Via extrinsic pathway
Endpoint	Clotting time (initiation phase)	Endogenous thrombin potential (initiation, propagation, and termination phase)
Results	APCsr **(Equation 1)**	nAPCsr **(Equation 2)**
Interpretation	Low APCsr indicates failure of APC to prolong the clotting time of plasma, defined as APCR	High nAPCsr indicates impaired down regulation of thrombin generation by APC, defined as APCR
Main determinants	FV Leiden FV levels FVIII levels Prothrombin levels	FV Leiden FV levels TFPI levels PS levels

Following the widespread use of this biomarker to evaluate the increased risk of VTE associated with COCs in the early 2000s, the Committee for Medicinal Products for Human Use (CHMP) of the European Medicines Agency (EMA) stated, in 2005, that APCR should be investigated during the development of new steroid contraceptives (European Medicines Agency, 2005). Nevertheless, the lack of standardization of this method has remained a problem for many years, and this has been reflected in highly variable results from one study to another, as depicted in **Figure 0.5**.

To overcome this issue, we recently developed and validated a new ETP-based APCR method aiming to provide a harmonized scale of nAPCsr. The analytical performances of this test are discussed in **Part I** with the validation of the methodology (**Part I.1**), the inter-laboratory variability (**Part I.2**), its implementation on a routine clinical equipment (**Part I.3**) and a comparison with the other APCR assays (**Part I.4**)**.**

In addition, the typical information obtained by thrombin generation investigation is available, providing much more information than the APCR itself. As it enables the assessment of the global coagulation process, this assay is also sensitive towards other factors of thrombogenicity like the prothrombin (PT) G20210A mutation, antithrombin and protein S deficiencies or FVIII levels (Duchemin et al., 2008; Lavigne-Lissalde et al., 2010; Marco et al., 2012; Szlam et al., 2011). The ETP-based APCR assay may thus provide information on haemostatic functions which are linked to an increased risk of thrombosis to help the prescriber in clinical decision-making.



The X-axis represents nAPCsr in absolute values and the Y-axis represents the studies included (first author, year, sample size). Studies are cited multiple times depending on the investigated COCs. Blue squares represent nAPCsr values (±SD) for "no-COC user" group ; green squares represent nAPCsr values (± SD) for "EE-LNG user" group; orange squares represent nAPCsr values (± SD) for "EE-DSG and EE-GSD user" group; red squares represent nAPCsr values (± SD) for "EE-DRSP user" group; pink squares represent nAPCsr values (± SD) for "EE-CPA user" group; purple squares represent nAPCsr values (± SD) for "E2/NOMAC or E2/DNG user" group and the gray square represents nAPCsr value (± SD) for "E4/DRSP user" group. The size of the square is related to the sample size. The clearest squares are chromogenic-based ETP-based APCR studies, and the darkest ones are Calibrated Automated Thrombogram-based studies. The validated and standardized ETP-based APCR assay is represented by a diamond.

Figure 0.5 | Synthesis of studies from 1997 to 2020 investigating the impact of COCs on the APCR when expressed as nAPCsr.

2009 - TOWARDS A SAFER ALTERNATIVE WITH OESTRADIOL-BASED COC

The oestrogen component remained unchanged for a long time, with the majority of COCs containing EE. Nevertheless, to further improve the tolerability of COCs and to broaden the choice for COC users, attempts have been made to replace EE with natural oestradiol (E2) (Ahrendt et al., 2009; Zeun et al., 2009).

The contraceptive efficacy of COCs is primarily derived from the action of the progestin compound but the oestrogenic moiety is also an important contributor to the efficacy of COCs. Indeed, oestrogens are partly responsible for suppressing the follicle-stimulating hormone, they potentiate the activity of the progestin component, by increasing progestin receptor concentration and they stabilize the endometrium so that irregular and unwanted bleeding can be minimized (Fruzzetti et al., 2010; Lobo et al., 1994). Although these desired effects on the reproductive organs, the occurrence of thrombotic events for which oestrogen was held responsible, led to a drastic reduction in EE dosage, as stated above.

However, reducing the amount of oestrogen, below 10µg of EE, with the aim to improve safety resulted in unacceptable changes in bleeding patterns compared to higher doses (Fruzzetti et al., 2010). Therefore, the development of COCs containing natural oestrogen, i.e., E2 was then suggested as an alternative of EE (Fruzzetti et al., 2010). The interest of using E2, as the oestrogen component in COCs was raised in the 1970s with the aim of improving the tolerability. Moreover, several studies showed that E2 impacted to a lesser extent the synthesis of hepatic protein compared to EE (Lindberg et al., 1989; Mashchak et al., 1982; Wiegratz et al., 2004). Because of its low oral bioavailability, E2 is either in a micronized form or esterified. Oestradiol valerate (E2V) presents similar pharmacokinetic and pharmacodynamic characteristics to that of E2 as it is rapidly converted to E2 in the intestines and the liver (1mg of E2V yields 0.75mg E2) (Medicines.org.uk, 2018). Indeed, the cleavage of E2V to E2 and the valeric acid occurs during absorption by the intestinal mucosa and in the course of the first pass effect. This gives rise to E2 and its metabolites oestrone (E1) and oestriol (E3) (Fruzzetti et al., 2010).

First E2-containing COCs, introduced in the 1980s, were monophasic preparations containing between 1 and 3mg of micronized E2. Although these preparations demonstrated effective ovulation inhibition and provided excellent contraceptive efficacy, these benefits were outweighed by unacceptable bleeding irregularities (Fruzzetti et al., 2010). An inappropriate oestrogen-progestin ratio or a suboptimal E2 dosage was pointed out as the plausible explanation for this failure in cycle control (Ahrendt et al., 2009; Fruzzetti et al., 2012). To address this issue, a preparation was developed in which E2V was combined with dienogest, in a four-phasic dosing regimen incorporating an oestrogen step-down and a progestin step-

up over 26 days (Klipping et al., 2011; Zeun et al., 2009). The treatment consists of the administration of E2V 3mg for 2 days – E2V 2mg/dienogest 2mg for 5 days – E2V 2mg/dienogest 3mg for 17 days – E2V 1mg for 2 days – and placebo for 2 days (Zeun et al., 2009). This dynamic dosing regimen was designed to ensure oestrogen dominance in the first part of the cycle and progestin dominance in the mid to late part of the cycle, thereby optimizing the control of bleeding (Borgelt et al., 2012). *Ahrendt et al.* demonstrated that this regimen certainly improved the cycle control and appeared to be associated with shorter and lighter bleeding compared with EE/levonorgestrel (Ahrendt et al., 2009). Furthermore, unlike other progestins, dienogest is a 19-nortestosterone derivative, with a 17-cyanomethyl instead of an ethinyl group at the C-17 position (Ruan et al., 2012) and possesses therefore a strong affinity for progesterone receptors, displays an antiandrogenic activity and lacks oestrogenic, glucocorticoid and androgenic properties. This suggested that this progestin would exert only minor metabolic effects (Junge et al., 2011). Studies of *Junge et al.* and *Klipping et al.* confirmed the minimal impact of this preparation on lipid, haemostasis, and carbohydrate metabolism (Junge et al., 2011; Klipping et al., 2011). This E2-containing COC became globally available in 2009 under the trade name Qlaira®. A few years later, in 2011, a new monophasic pill containing 1.5mg of micronized 17β-E2 and 2.5mg nomegestrol acetate was introduced in the European market, under the trade name Zoely®, and consisted of a 24-day regimen followed by 4 days of placebo (Christin-Maitre et al., 2013). Nomegestrol acetate is a progesterone derivative, and more specifically, a 19-norpregnane, possessing an antiestrogenic activity on the endometrium and a moderate antiandrogenic activity. Through this enhanced selectivity profile, this preparation was expected to provide acceptable cycle control and limit cardiovascular and metabolic side effects. This was confirmed by *Agren et al.* and *Gaussem et al*. who revealed that E2/nomegestrol acetate had a similar safety and tolerability profile to EE/levonorgestrel (Agren et al., 2011; Gaussem et al., 2011). As these studies were based on biological and pharmacological data with no attempts to correlate haemostatic changes with VTE risk, epidemiological data on the risk of VTE associated with these E2-containing COCs were requested by the regulatory agencies. Two large international active surveillance studies, the INAS-SCORE (NCT01009684) and the PRO-E2 (NCT01650168) were initiated to assess the risk of short- and long-term use of E2V/dienogest and E2/nomegestrol acetate respectively (Jensen et al., 2013; Reed et al., 2021). In comparison with COC-levonorgestrel users, the incidence of VTE was slightly lower in users of E2V/dienogest and E2/nomegestrol acetate (**Table 0.3**). This suggests that EE/levonorgestrel is not the only option for minimizing the risk of VTE associated with COC use, but E2-containing products are equally safe (Dinger et al., 2016). This also reflects that those biological investigations may, at least in part, correlate with epidemiological data. This also permits to put the pharmacodynamics

data requested by the EMA during the development of steroid contraceptives into a more clinical context.

Table 0.3 | Estimated risk of VTE with COCs

Risk group	*Estimated risk of VTE*	*Reference*
Non-pregnant nonuser	2/10 000 WY	EMA/739865/2013 (European Medicines Agency, 2014)
Pregnant and postpartum women	20/10 000 WY	30-year population-based study (Heit et al., 2005)
User of COC containing **- EE + LNG** **- EE + NETA** **- EE + NGM**	5-7/10 000 WY	EMA/739865/2013 (European Medicines Agency, 2014)
User of COC containing **- EE + GSD** **- EE+ DSG** **- EE+ DRSP**	9-12/ 10 000 WY	EMA/739865/2013(European Medicines Agency, 2014)
User of COC containing **- EE + CMA**	Unknown	N/A
User of COC containing **- EE + DNG**	8-11/10 000 WY	DHPC Louise (CeresPharma, 2021)
User of COC containing **- E2V + DNG**	7.0/10 000 WY versus 3.5 in nonusers and 9.9 in LNG/EE users	INAS-SCORE study (Jensen et al., 2013)
User of COC containing **- E2/NOMAC**	2.0/10 000 WY versus 1.8 in nonusers and 3.0 in LNG/EE users	PRO-E2 (Reed et al., 2021)
User of COC containing **- E4/DRSP**	Pending	PASS required by the EMA and FDA

2013 - THE EUROPEAN MEDICINES AGENCY REFERRAL: WHAT NEEDS TO BE DONE?

In 2013, France informed the EMA, pursuant to Article 31 of Directive 2001/83/EC, of their consideration that the benefit-risk balance of CHCs had become unfavourable in the currently authorized indication due to the increased risk of VTE. The Pharmacovigilance Risk Assessment Committee (PRAC) was then requested to make a recommendation on whether the indication of medicinal products containing chlormadinone acetate, desogestrel, dienogest, drospirenone, etonogestrel, gestodene, norelgestromin, norgestimate, and nomegestrol acetate should be restricted and/or any other regulatory measures taken. In the context of this referral, all pharmacoepidemiology studies on CHCs were reviewed to assess the estimated risk of VTE associated with each CHC preparation **(Table 0.3).** Oral contraceptives considered as the safest in terms of VTE risk were preparations containing EE associated with either levonorgestrel, norethisterone acetate or norgestimate. The

highest estimated incidence of VTE was observed with COCs containing EE associated with gestodene, desogestrel or drospirenone (i.e., 9-12 VTE/10,000 women a year) but compared with pregnancy and postpartum period (i.e., 20 VTE/10,000 women a year), it remained lower (European Medicines Agency, 2014; Heit et al., 2005). It was also highlighted that the risk of VTE was higher during the first year of use or following a restart after a one-month period without treatment (European Medicines Agency, 2014).

As a risk minimization strategy, the PRAC considered that modifications in the summary of product characteristics (SmPC) were required to strengthen information relative to the associated risk of VTE. In addition, they recommended the implementation of educational measures to increase healthcare professionals and women awareness regarding the contraindications and the risk factor of VTE and they mentioned that the individual's risk should be re-evaluated periodically as a risk factor for VTE can change over the course of a lifetime (European Medicines Agency, 2014). On this basis, several guidelines offer support in tailoring contraceptive therapies, according to the patient's profile. There are the US Medical Eligibility Criteria for contraceptive use (CDC, 2016), the World Health Organization Medical Eligibility Criteria for contraceptive use (Fifth Edition, 2015) (World Health Organization, 2015) and the UK Medical Eligibility Criteria for contraceptive use (UKMEC 2016) (FSRH, 2016).

2020 - THE CHALLENGE OF IDENTIFYING WOMEN AT HIGHER RISK OF VTE, DEPENDING ON THEIR HORMONAL STATUS

Many factors must be considered and discussed with the women when choosing a contraceptive method. In addition to the efficacy, the tolerability and the additional health benefit, the risk of VTE is an important element that must be evaluated. At the present time, this risk is only assessed based on clinical characteristics and does not rely on formal algorithms including phenotypic coagulation screening with laboratory tests. The standard of care, when starting contraception, consists of identifying the presence of hereditary thrombophilia solely on the basis of familial history of VTE; a strategy which has been reported to be of low sensitivity and predictive value (Cosmi et al., 2003; Suchon et al., 2016). Indeed, identifying a thrombosis within the family does not necessarily mean that there is an underlying thrombophilia. Genetic risk factors alone contribute to only 30% of the family history of VTE (Hannaford, 2011). Moreover, the exclusion of the main hereditary thrombophilia does not mean that a woman will not suffer from a thrombotic event under COC use (Hannaford, 2011). Venous thromboembolism is a multifactorial disease whose occurrence depends on the interaction between gene defects and environmental factors (**Figure 0.2**) (Cosmi et al., 2003). As a result, exposure of high-

risk situations such as surgery, trauma, immobilization (e.g., casts, long-range travel/flights), pregnancy or hormonal therapy may trigger a thrombotic event in individuals either in absence or presence of genetic mutations, suggesting that the evaluation should be on the phenotypic rather than the genotypic thrombophilia expression.

There are five well-known genetic thrombophilia that can be divided in two main categories: gain of function mutations and loss of function mutations. Mutations conferring a gain of function include FVL and PT G20210A mutations, which are the more frequent genetic risk factors observed in the Caucasian population. The prevalence reaches 5% for FVL mutation and 2% for PT G20210A mutation. In contrast, they are much rarer in African and Asian populations (Hotoleanu, 2017). The risk of first VTE event is 3- to 7-fold higher in heterozygous carriers while it may reach a relative risk of 30 to 80 in homozygous carriers (Mannucci et al., 2015). Mutations that confer a loss of function, concern deficiencies of protein C, protein S and antithrombin. These mutations are less frequent with prevalence below 1% but they are associated with a 10- to 50-fold risk of first VTE (Mannucci et al., 2015). The presence of one of the above mutations with COCs leads to a synergistic and amplificative (rather than an additive) prothrombotic effect. *Hugon-Rodin et al*. and *Khialani et al.* investigated the joint effect of COCs and genetic mutation, e.g., FVL or PT G20210A mutations. Both research groups have calculated a synergy index (SI), reflecting the amplificative effect of the combination of a genetic mutation with COCs above the simple addition of the independent risk alone (Hugon-Rodin et al., 2018; Khialani et al., 2020). Based on this, *Khialani* and co-workers estimated the odds ratios at 19.3 and 24.0 in carriers of FVL mutation and PT G20210A mutation when using the pill, respectively (Khialani et al., 2020).

Consequently, there is a need of being able to identify the baseline risk of VTE in women before introducing hormonal contraceptives (Morimont, Dogné, et al., 2020). In 2015, Gene Predictis® launched the Pill Protect® on the Swiss market (Michaud et al., 2016). This diagnostic device is based on an algorithm which considers nine polymorphisms and four clinical risk factors associated with VTE development as well as the potential COC that could be prescribed **(Table 0.4).**

To assess the performance of this test, a comparison was performed to the "current" practice, which was based on oral anamnesis of the patient, and to the genotyping for FVL and PT G20210A mutations.[1] Result of the ROC curve analyses, reflected by the area under the curve, was higher (i.e., 0.71) with the Pill Protect® compared to

[1] It has to be noted that the genotyping of FVL and PT G20210A mutations is not part of the current practice.

the oral anamnesis of the patient (i.e., 0.61) or the simple genotyping of FVL and PT G20210A mutations (i.e., 0.67).

Table 0.4 | Clinical and genetic parameters assessed with the screening test Pill Protect®

Clinical variables	Age	
	BMI	
	Smoking habits	
	Family history of VTE	
Genetic variables	Gene: F5 SNP: rs6025 Allele: A	Gene encoding for coagulation factor V. The resulting re6025(A) allele is known as Factor V Leiden mutation which leads to some resistance to the APC and an increased risk of thrombosis (McDaid et al., 2017).
	Gene: F2 SNP: rs1799963 Allele: A	Gene encoding for prothrombin. The resulting rs199963(A) allele is known as G20210A mutation which leads to increased plasma prothrombin levels and an increased risk of thrombosis (McDaid et al., 2017).
	Gene: ABO SNP: rs8176719 Allele: G SNP: rs8176750 Allele: C	Gene encoding for ABO subtypes. The rs8176719(G) and rs8176750(C) alleles encode for non-O blood groups and are associated with an increased risk of VTE through modifications of von Willebrand Factor (VWF) and factor VIII (FVIII) plasma levels (Morange et al., 2013).
	Gene: F11 SNP: rs2289252 Allele: T	Gene encoding for coagulation factor XI. The rs2289252(T) allele is associated with increased FXI activity leading to a procoagulable state (Morange et al., 2013).
	Gene: CYP2C9 SNP: rs1799853 Allele: T	Gene encoding for cytochrome CYP2C9 involved in the metabolism of EE. The rs1799853(T) allele could induce a decrease in the metabolism of EE, thus increasing its plasma levels and therefore the global estrogenicity (McDaid et al., 2017).
	Gene: PROCR SNP: rs9574 Allele: G	Gene encoding for APC receptors involved in the activation of the anticoagulant pathway. The rs9574(G) allele has been reported to be at increased risk of VTE compared to the C allele (Morange et al., 2013).
	Gene: SUGCT SNP: rs4379368 Allele: T	Gene encoding for the succinate-hydroxymethylglutarate CoA-transferase. The rs4379368(T) allele has been associated with migraine susceptibility. The combination of both migraine and COC could further increase the risk of cardiovascular diseases (Kaur et al., 2019).
	Gene: KNG1 SNP: rs710446 Allele: C	Gene encoding for kininogen-1. This protein plays an important role in the coagulation process by assembling the kallikrein-kinin system. The rs710446(C) allele has been associated with shortened aPTT levels and an increased risk of VTE (Morange et al., 2011).

These data revealed the usefulness of this prognostic device and reopened the reflection of performing biological investigations before the introduction of a contraceptive method (McDaid et al., 2017). Although this method is promising, the algorithm does not take into account a deficiency of protein S, protein C or antithrombin and in addition, many users of COCs developing VTE do not have a recognized hereditary coagulation problem but show instead a high responsiveness to oestrogenic compounds (Douxfils, Morimont, & Bouvy, 2020). An interesting approach would be to have one or several biomarkers to establish the "coagulability status" of the patient, revealing phenotypic rather than just genotypic particularities.

Global sensitive assay like the ETP-based APCR assay could be potential candidates. One rationale and affordable perspective could be the screening of the coagulability state to provide objective data for the gynaecologist to support the prescription of the most appropriate contraceptive method. In case of an abnormal result, which suspects the presence of an underlying pathology, the women can be referred to a haematologist for further investigations. This is of particular importance since the identification of coagulopathies may drive the behaviour of both the patient and the healthcare professional for the entire lifetime of the woman. Subsequently, a second testing after a sequence of at least one cycle of hormonal treatment would allow identifying women with an abnormal rise of the nAPCsr which may reveal an oversensitivity to the oestrogenic effect of COC or particularities in the metabolism of the concerned COC.

A global screening test would represent a more appropriate and cheaper alternative than a full thrombophilia test panel for assessing the risk since, this test does not only focus on the inherited coagulopathies but also assesses the individual sensitivity towards COCs. We believe the ETP-based APCR could be a solution and this hypothesis is addresses in **Part II**.

Furthermore, information on the coagulability status could not only reduce the risk of COC-induced thrombosis but also the incidence of thrombotic events in situations associated with elevated thrombotic risk. It has to be reminded that VTE and pulmonary embolisms are associated with a significant mortality but also with a high morbidity rate leading to expense costs for the diagnosis, the treatment, and the management of any thrombotic related disability such as recurrent VTE, post-thrombotic syndrome or chronic pulmonary hypertension. All of these lead to severe impairment of the affected women, an important financial burden within personal expenses, healthcare resources and societal costs (Morimont, Dogné, et al., 2020). Obviously, such strategies need to be evaluated by proper epidemiological and cost-effectiveness studies but with the advent of new technologies permitting the global assessment of the coagulation process, there is a new era for management of

women willing to get the most appropriate and safe contraceptive method based on their individualized profile.

2021 - A NOVEL COMBINED ORAL CONTRACEPTIVE BASED ON A NATIVE OESTROGEN, ESTETROL

Estetrol (E4) was discovered in the mid-1960s by Diczfalusy and co-workers by investigating the metabolism of E2 in early pregnancy (Coelingh Bennink et al., 2008). This steroid molecule, characterized by 4 hydroxyl groups, is exclusively synthesized in the foetal liver which is the only organ capable of both 15α- and 16α-hydroxylation**.** Estetrol is present in maternal blood and urine from the ninth week of gestation and reaches the maternal circulation through the placenta (Holinka et al., 2008). Produced in increasing quantities during the foetal lifespan, it was suggested that E4 could be a safe oestrogenic steroid for human use. Pharmacokinetic studies demonstrated an interesting profile with a good oral bioavailability, no metabolization into active metabolites, i.e. E3, E2 or E1, and a half-life time around 28 hours, suggesting suitability for once daily administration (Coelingh Bennink et al., 2008). From a pharmacodynamic point of view, E4 showed a high selectivity for the oestrogen receptors (ER) and weak interactions with glucocorticoid, progesterone and testosterone receptors (Coelingh Bennink et al., 2008). The binding affinity for both oestrogen α and β receptors (ERα and ERβ) was moderate with a 4- to 5-fold higher affinity for ERα. Like the other oestrogens, E4 activates the nuclear ERα but in contrast with other oestrogens, it antagonizes the activity of membrane ERα involved in more rapid signalling pathways. Oestrogens can act through this distinctly different pathway by inducing rapid extra-nuclear activity via a small pool of ERα located close to the membrane. This process is defined as membrane-initiated steroid signalling (MISS) and may result in the activation of intracellular signalling pathways (e.g., PI3K, MAPK), the activation of multiple kinases and the production of a variety of downstream second messengers (e.g., nitric oxide, calcium flux, cyclic adenosine monophosphate), directly influencing cell activities that contribute to the regulation of cell survival and proliferation. Interaction of nuclear and MISS pathways remains to date poorly recognized, but the kinases activated by the MISS pathway can, in turn, phosphorylate various transcription factors, including ERs and coregulators, and therefore indirectly modulate the transcriptional activity in the nucleus **(Figure 0.6)** (Abot et al., 2014; Arnal et al., 2017; Gérard et al., 2015; Zhang et al., 2006).

Animal and human studies demonstrated that E4 behaves as an agonist in bones, uterus, and brain (hot flush, ovulation inhibition, etc.) through nuclear ERα but as an antagonist of ERα-dependent MISS pathway, and especially in the endothelium

(considered as one of the NO synthase activation pathways) (Abot et al., 2014; Coelingh Bennink et al., 2008; Valera et al., 2018)..

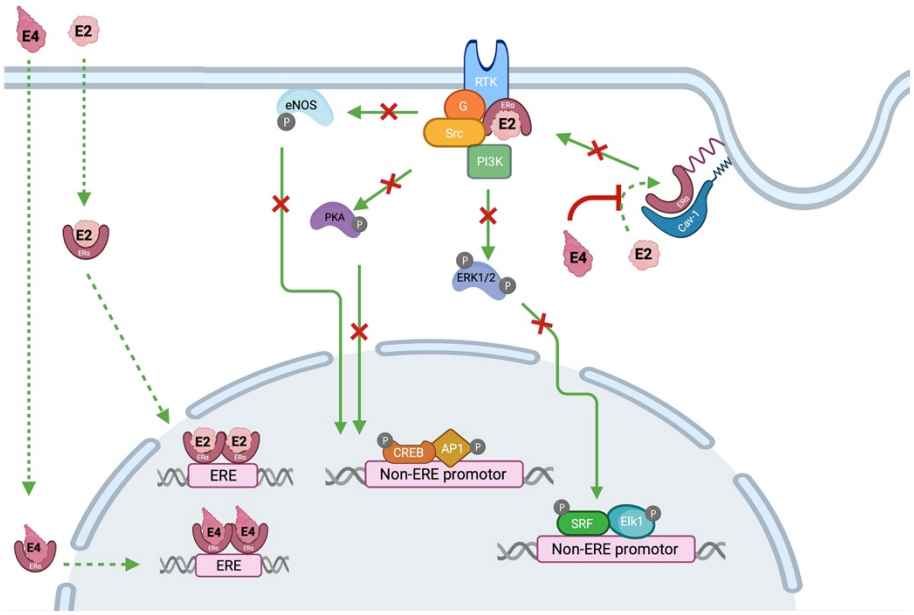


Figure 0.6 | Interaction between oestrogens, i.e., E2, and E4, and oestrogen-receptor alpha

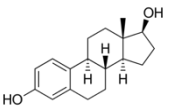
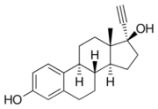
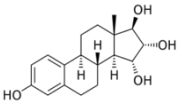
Oestradiol activates both membrane and nuclear actions of ERα while E4 is an agonist of nuclear activity but an antagonist of the ERα-dependent MISS pathway. Nuclear activity is the results of an interaction between oestrogens, i.e., E2 or E4, and the ERα located in the cytoplasm. This binding leads to the dimerization and the translocation of the complex into the nucleus, where it interacts with oestrogen response element (ERE) DNA sequences in target genes. The ERα-dependent MISS pathway consists of a rapid nongenomic activity playing an important role in the endothelial effect of oestrogens. Palmitoylation of ERα allows them to anchor to the plasma membrane caveolae where they associate with caveolin-1 (Cav-1). Upon E2 stimulation, ERα is de-palmitoylated and dissociated from Cav-1, to interact with protein kinase (Src and PI3K), G-coupled protein ai (Gai) leading to signalling cascade (Akt, Pka, ERK1/2) and endothelial NO synthase activation. On the other hand, E4 is devoid of ERα MISS activity and even is also able to antagonize E2-induced MISS effect, especially in the endothelium.

With this mode of action, E4 has been recognized as a New Active Substance (NAS) by the EMA and can be described as the first Native Oestrogen with Specific action in Tissues (NEST), a classification which differs from the selective oestrogen receptor modulators (SERM) (Foidart et al., 2019). A pharmacological comparison between E4, EE and E2 is shown in **Table 0.5.**

These pharmacological properties made E4 an appropriate candidate for contraception and menopausal replacement therapy. Based on pre-clinical and clinical data, multiple E4/drospirenone and E4/levonorgestrel dose combinations were investigated and compared to 20µg of EE in association with 3mg of drospirenone, for their effects on ovulation inhibition and haemostatic biomarkers

(Mawet et al., 2015). Regardless of the dose, i.e., 5mg, 10mg, or 20mg of E4 and the progestin, i.e., either levonorgestrel or drospirenone, E4-containing COCs effectively blocked ovulation and induced minor effects on haemostasis markers (Mawet et al., 2015).

Table 0.5 | Comparison of E2, EE and E4

		Oestradiol (E2)	*Ethinylestradiol (EE)*	*Estetrol (E4)*
Origin		Natural Synthesized in the growing ovarian follicle, corpus luteum, placental, adrenal and Leydig cells, liver, endometrium, brain muscle and fat tissue.	Synthetic derivative	Natural Exclusively synthesized in the fetal liver
Chemical structure				
Dosage in COC		1-3mg	10-50µg	15mg
Associated progestin in COC		NOMAC DNG	LNG, NETA, NGM, DSG, GSD, DRSP, CPA, DNG, CMA	DRSP
PK profile	**Oral bioavailability**	Low oral bioavailability but administered either micronized or esterified.	Good oral bioavailability	Good oral bioavailability
	Metabolism	High metabolism into E1 (sulfate) and E3 (sulfate)	High metabolism into various conjugates (glucuronidated, sulfated and hydroxylated metabolites)	No metabolization
	Half-life time	Half-life time around 35 hours (E1 serves as a precursor of E2 and can be transformed back into E2)	Half-life time around 12 hours	Half-life time around 28 hours
PD profile			High selectivity for ER (higher affinity for ERα)	High selectivity for ER (higher affinity for ERα); First Native Estrogen with Specific action in Tissues (NEST)
Impact on liver protein synthesis		Minor (non-negligible contribution of E1)	Major	Minor

In the meantime, an open-label, multi-centre, randomized, dose-finding study (FIESTA) was performed to assess bleeding pattern and cycle control of E4 combined with either drospirenone or levonorgestrel (Apter et al., 2016). The combination of 15mg E4 with 3mg drospirenone proved to be the most efficacious with respect to bleeding and cycle control and showed the most satisfaction among the users (Apter et al., 2016; Apter et al., 2017). Further haemostasis investigations revealed that this novel E4-containing COC had a very low impact on the coagulation and fibrinolytic systems (Douxfils, Klipping, et al., 2020). Details on the phase II clinical trial regarding the impact on haemostatic parameters, APCR and thrombin generation are found in **Part III.**

In light of the clinical efficacy and safety data from the Phase II program, the association of E4 at the dose of 15mg with 3mg of drospirenone (monophasic 24+4 regimen) was selected for phase III development (Apter et al., 2016). The E4 Female Response concerning Efficacy and safety of Estetrol/Drospirenone as Oral contraceptive in Multicentric study (FREEDOM) Phase III program consisted of two open-label, single arm studies, one performed in Europe and Russia and the other one in the US and Canada totalling 3,725 women. The studies confirmed the contraceptive efficacy, a good bleeding profile and cycle control and also reported a high satisfaction rate (Grandi et al., 2020). This led to the approval of E4/drospirenone by several regulatory agencies since the beginning of 2021 (European Medicines Agency, 2021).

CONCLUSION

Over the last 60 years, efforts have been made to reduce the risk of VTE events associated with COCs, and today, all strategies seem to be moving towards the safe use of these products. With novel formulations on the market, i.e., E2- and E4-based COCs, the association of EE with levonorgestrel should no longer be the only option for minimizing the risk of VTE associated with COCs use. In addition to the development of safer products, attempts are being made to improve the management of patients who desire to start a contraceptive therapy. The proposal of a global screening test before the initiation of a contraceptive therapy could significantly reduce the 22,000 cases of thrombosis observed each year in Europe following the use of COCs (McDaid et al., 2017).

PART I: ANALYTICAL PERFORMANCES OF THE ETP-BASED APC RESISTANCE ASSAY

Clinical Chemistry Laboratory Medicine. 2020; 58(2)

I.1 - VALIDATION AND STANDARDIZATION OF THE ETP-BASED ACTIVATED PROTEIN C RESISTANCE TEST FOR THE CLINICAL INVESTIGATION OF STEROID CONTRACEPTIVES IN WOMEN: AN UNMET CLINICAL AND REGULATORY NEED

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SUMMARY

Background: Regulatory bodies recommend the use of an assay based on the assessment of the endogenous thrombin potential (ETP) for the investigation of the activated protein C resistance (APCR) in the development of steroid contraceptives in women. However, the assays described in the literature are home-made and not standardized regarding the method, the reagents, the reference plasma, and the quality controls.

Aims: In the absence of any commercially available method, we aimed at validating the ETP-based APCR assay.

Methods: The validation was performed according to regulatory standards. The method targets a 90% inhibition of the ETP in healthy donors in presence of APC compared to the same condition in the absence of APC. As a large-scale production of a pool of plasma from well-selected healthy donors is impossible, algorithms are applied to a commercial reference plasma to correlate with the selected pool.

Results: Repeatability and intermediate precision passed the acceptance criteria. The assay demonstrated a curvilinear dose response to protein S and APC concentrations (R^2>0.99). Analysis of plasma samples from 47 healthy individuals (22 women not taking combined hormonal contraceptives and 25 men, no factor V Leiden carrier) confirmed the validity of the test, with a mean inhibition percentage of 90%. Investigations in 15 women taking different contraceptives and in 2 subjects with FV Leiden mutation confirmed the good sensitivity and performance of the assay.

Conclusion: This validation provides the pharmaceutical industry, the regulatory bodies, and the physicians with a reproducible, sensitive, and validated gold-standard ETP-based APCR assay.

INTRODUCTION

The Committee for Medicinal Products for Human Use (CHMP) of the European Medicines Agency (EMA) has issued in 2005 a guideline on clinical investigation of steroid contraceptives in women. As clinical trials generally include an insufficient number of women to provide information on cardiovascular events or venous thromboembolism (VTE), this guideline recommends the biological variables possibly related to different VTE risk to be investigated (European Medicines Agency, 2005). Among these markers, the endogenous thrombin potential (ETP)-based activated protein C resistance (APCR) is recommended. The measurement of the APC resistance (APCR) using the ETP has been first described in 1997 by Nicolaes et al. (Nicolaes et al., 1997). The test was developed to assess the acquired resistance to APC in pregnant women, women taking oral contraceptive or menopausal replacement therapy and in subjects with protein S deficiency, carrying a factor V Leiden (FVL) or a prothrombin (PT) G20210A mutation (Curvers et al., 1999; Curvers, Thomassen, et al., 2002; Rosing et al., 1999). The normalized APC sensitivity ratio (nAPCsr) was then defined as the ETP ratio in presence and in absence of exogenous APC of the patient's sample divided by the same ratio from a reference plasma (Nicolaes et al., 1997).

The test was initially performed on a thrombin generation system based on the cleavage of a chromogenic substrate specific for thrombin (Nicolaes et al., 1997). Because technical and methodological difficulties limited this technique (Tchaikovski et al., 2007) and because of the technological advance brought by the calibrated automated thrombogram (CAT) of Hemker et al. (Hemker et al., 2003), the ETP-based APCR assay was adjusted on this fluorometric technique (Brugge et al., 2006; Tchaikovski et al., 2007). However, the limitations of the CAT system, such as the lack of standardization of the reagents, the absence of quality controls and reference plasma and the batch-to-batch variation, are also applicable to the ETP-based APCR assay (Dargaud et al., 2010; Dargaud et al., 2007; Loeffen et al., 2012; Perrin et al., 2015). Indeed, besides the differences imposed by a chromogenic or a fluorogenic assay, with the use of defibrinated plasma or platelet poor plasma (PPP) respectively, differences in the source and concentration of tissue factor (TF), APC and phospholipid (PLs) vesicles led to variable sensitivity of the assays towards the APCR (Brito et al., 2012; Johnson et al., 2008; Lebreton et al., 2017; Raps et al., 2012; Raps et al., 2013; Tans et al., 2003; Tchaikovski et al., 2007; Westhoff et al., 2017). This hampered study-to-study comparisons which *in fine* impeded the proper evaluation of the safety of hormonal therapies or evaluation of prothrombotic states. In the absence of any commercially available method approved by competent authorities, the validation of this assay was a requirement for the assessment of ETP-based APCR

in clinical studies (European Medicines Agency, 1995; Food and Drug Administration, 2018).

The aim of this study was to validate the ETP-based APCR assay using commercially available reagents to ensure batch-to-batch traceability, recovering and reproducibility of the method over time. The validation was performed according to the best industry standards including FDA "Guidance for Industry: Bioanalytical Method Validation" and ICH Q2(R1) "Validation of Analytical Procedures: Text and Methodology" (European Medicines Agency, 1995; Food and Drug Administration, 2018).

MATERIALS AND METHODS

The study protocol was in accordance with the Declaration of Helsinki and was approved by the Medical Ethical Committee of the Centre Hospitalier Universitaire (CHU), Université Catholique de Louvain (UCL) Namur, Godinne (Yvoir, Belgium) under the number B03920096633. A written informed consent was obtained from each donor. This study was carried out at QUALIblood facilities, a contract research organization in Namur, Belgium.

Plasma collection of healthy donor's sample

Procedure of blood sampling

Blood was taken by venepuncture in the antecubital vein and collected into 0.109 M sodium citrate (9:1 v/v) tubes (Vacuette®, Greiner, Austria) without corn trypsin inhibitor using a 21-gauge needle (Terumo). The first tube was always discarded, and the first centrifugation was performed within 30 min. The PPP was obtained from the supernatant fraction of blood tubes after a double centrifugation for 15 min at 2,500 x g at room temperature. The centrifuge brake was set to the minimum position at the end of the process. The residual platelet count was measured to ensure the centrifugation procedure provides plasma with a platelet count <10.000 platelets/µL. Immediately after centrifugation, PPP was aliquoted by 1 mL or pooled for the constitution of the in-house reference plasma and aliquoted by 4 mL. The aliquots were then snap-frozen in liquid nitrogen before being stored at ≤ -70°C. Frozen PPP samples were thawed, heated to 37°C for 2-3 min and mixed gently just before the experiment. All tests were performed within 4h after thawing.

Population for the constitution of the normal pooled plasma, for the definition of the normal range and from women taking hormonal contraceptives and/or carriers of factor V Leiden mutations.

Twenty healthy donors (10 males and 10 females) were enrolled to constitute the in-house healthy pooled plasma (HPP), later called the in-house reference plasma. The

mean age of the participants was 23 years old (range: 18–47 years old) and the mean body mass index (BMI) was 22.3 kg/m^2 (range: 16.3-29.0 kg/m^2). Exclusion criteria were history of thrombotic and/or haemorrhagic events, treatment by an anti-platelet or anticoagulant medication or other drug potentially affecting platelets or coagulation factors, pregnancy, and use of hormonal therapy (i.e., contraceptive or menopausal replacement therapy). These donors were not carriers of FVL or PT G20210A mutations.

For the definition of the normal range, 50 healthy individuals (22 females and 28 males) were initially included but 3 males were excluded due to incomplete clinical data provided in the questionnaire. The exclusion criteria were the same as above. The mean age was 25 years old (range: 18–58 years old) and the mean BMI was 22.8 kg/m^2 (range: 16.3-30.3 kg/m^2).

To assess the stability of the sample for the ETP-based APCR assay over time, 6 plasma samples from healthy males and 6 plasma samples from women under contraceptives were collected and stored as above. These samples were also used for the first batch changing to ensure between batch reproducibility according to the CLSI-EP26-A document (Clinical and Laboratory Standards Institute, 2013).

To assess the sensitivity of the test in a small subset of random real-life samples, 15 healthy young women taking hormonal therapy since at least 3 cycles were recruited. The exclusion criteria were the same as above. Women were stratified in 3 groups, i.e., 5 women taking 2nd generation combined oral contraceptives (COC), 5 women taking 3rd generation COC and 5 women taking either a progestin-only oral contraceptive or an implant (two on desogestrel oral, one on nomegestrol oral and two on etonogestrel implants). Besides, two samples from young women, both carriers of a heterozygote FVL mutation, were tested. One of these two young women was also treated with a 2nd generation COC. These two FVL mutations were confirmed by a CE-approved technique for the detection of FVL G1691A genetic polymorphism (LaCAR MDx Technologies S.A., Liège, Belgium). The FVL mutation in the young women not on COC was also confirmed by a coagulation test with a ratio of 1.53 (reference range value [ratio]: negative sample ≥ 2.9; heterozygote: 1.3–1.8; homozygote: 0.9–1.1) (APC Resistance kit, Technoclone, Vienna, Austria).

General procedure of the ETP-based APCR assay

The ETP-based APCR assay was assessed on the CAT (Thrombinoscope BV, Maastricht, The Netherlands) with the Thrombinoscope software (Thrombinoscope BV, version 5.0). The concentration of APC was determined to ensure a 90% inhibition of the ETP (***Equation 3***) of the in-house reference plasma. In the literature, the ETP-based APCR is usually expressed as a "normalized APC sensitivity ratio"

(nAPCsr) (as already described in ***Equation 2***). The present study will compare these two expressions of the results using samples from the entire population included.

Equation 3 :

$$\text{Inhibition \%} = 1 - \frac{Sample\ ETP\ (+APC)}{Sample\ ETP\ (-APC)}, expressed\ in\ \%$$

Triggering reagents

Measurements were conducted using commercially available CE-marked thrombin generation dedicated kit reagents from Diagnostica Stago (Diagnostica Stago, Asnières-sur-Seine, France). The activator reagent, the STG-ThromboScreen -TM, is a mixture of PLs (± 4µM) and TF (± 5pM) in which a fixed concentration of APC (Enzyme Research Laboratories, Swansea, United Kingdom) was introduced for the APC-positive (+APC) condition. The fluorogenic substrate was brought by the FluCa-kit (Diagnostica Stago) which contains calcium chloride at 160mM to trigger the coagulation cascade. Each well was filled up with 20µL of activator reagent, in absence or in presence of APC, and 80µL of plasma. A 10 min incubation at 37°C in the fluorometer was performed before injecting 20µL of FluCa-Kit to start measurements.

It is worth mentioning that, as the manufacturer fulfils the batch-to-batch comparability testing requirements described in the CLSI-EP26-A document, the concentration of TF and PLs may slightly vary from batch-to-batch (Clinical and Laboratory Standards Institute, 2013). For this reason, the complete activation/triggering system has to be validated for each batch of reagents. For each new batch combination of reagents, the concentration of APC required to obtain a 90% of inhibition of the ETP in the +APC condition compared to the -APC condition has to be determined. Thus, the concentrations provided in the following section are only informative.

Reference plasma

The use of reference plasma is dedicated to the normalization of the thrombin generation test (TGT) parameters and to the determination of APC concentration. Two different reference plasmas were used for the validation: the in-house reference plasma (*see plasma collection of healthy donors' samples for more details)* for the determination of the APC concentration to obtain the initial 90% of inhibition of the ETP and one commercially available reference plasma, STG-Ref Plasma TS, dedicated to a thrombin generation application (Diagnostica Stago) for experiment-to-experiment normalization and to ensure batch-to-batch recovery. Note that another cryogenized reference plasma (CRYOcheck Pooled Normal Plasma; Cryopep, Montpellier, France) was also used for mixing studies with immunodepleted protein

S plasma (Cryopep) certified as containing less than 1% of protein S for antigenic and functional dosage.

Quality controls

Three levels of quality controls (QC) were used. These were lyophilized citrated human plasma designed for thrombin generation testing (Diagnostica Stago) and described as hypocoagulable (STG QualiTest Low TS, QC low), intermediate (STG-QualiTest Norm TS, QC norm) or hypercoagulable (STG-QualiTest High TS, QC high). These controls provided 3 distinct levels of response in the ETP-based APCR assay.

Validation of the ETP-based APCR assay

The validation was performed according to the "best industry standards" (European Medicines Agency, 1995; Food and Drug Administration, 2018). The validation steps and their corresponding acceptance criteria to be fulfilled to ensure validation of this assay are listed in **Table I.1.1**.

Table I.1.1 | Acceptance criteria and results for the different validation steps.

	Acceptance criteria	Results
Linearity		
- **Activated Protein C dose response**	- R^2>0.99 (non-linear regression accepted)	- R^2=0.996 (for in-house reference plasma)
- **Protein S deficiency dose response**	- R^2>0.99 (non-linear regression accepted)	- R^2=0.998
Stability of reagents	- All points (3 levels of QCs and the commercial reference plasma) must be within the acceptance range calculated as the mean inhibition of the 4 manipulations expressed in % ±5% for each level of control	- All QCs were within 5% of deviations of the mean percentage of inhibition during the 4-hour period.
Precision – Repeatability		
- **Intra-run repeatability**	- SD < 10%	- SD were 0%, 1%, 3% and 0% for the QC low, intermediate, high and the commercial reference plasma.
- **Inter-run repeatability**	- SD < 10%	- SD were 0%, 7%, 4% and 3% for the QC low, intermediate, high and the commercial reference plasma.

Table I.1.1 (continued) | Acceptance criteria and results for the different validation steps.

	Acceptance criteria	Results
Precision – Intermediate precision	- SD < 10% and no significant difference between operators (evaluated by ANOVA with Tukey's multiple comparison test). A p-value below .05 considers the difference between operators as significant.	- The SD of the 3 different operators range from 0% to 0%, 2% to 5%, 0% to 4% and 1% to 2% for the QC low, intermediate, high and the reference plasma respectively. The p-values were .8503, .6969, .8253 and .9459 for the QC low, intermediate, high and the reference plasma respectively.
Reference range	- Mean percentage of inhibition of the 47 healthy donors* = 90 ± 2.5%.	- The mean percentage of inhibition of the 47 healthy donors was 90% (SD = 6%; 95% confidence interval of the mean = 89% to 92%; 10^{th} – 90^{th} percentile = 82% - 100%).

*Three donors were excluded due to incomplete information provided on the informed consent.

Linearity analysis

The linearity was assessed by dose-response curves performed with (i) various concentrations of exogenous APC added to the triggering reagent and (ii) with tested plasma containing different levels of protein S. For assays with exogenous APC, reference plasmas were compared (i.e., the in-house reference plasma and the commercial reference plasma). The different protein S concentrations were obtained by mixing a cryogenized immuno-depleted protein S deficient plasma (Cryopep) with a commercially available cryogenized reference plasma (CRYOcheck Pooled Normal Plasma, Cryopep) at different proportions (tested ratio protein S deficient plasma/CRYOcheck: 100/0; 90/10; 80/20; 60/40; 40/60; 20/80; 10/90 and 0/100). The percentage of protein S contained in the different mixes was assessed on a STA-R MAX analyser (Diagnostica Stago) using the STA®-Staclot® Protein S assay according to the manufacturer recommendations.

Stability of the reagents

To ensure that reagents are stable after reconstitution in case of reagent re-use, stability of the reagents has been assessed 1h, 2h, 3h and 4h after reagent reconstitution and stabilization (i.e., 30 min) at room temperature. The 3 levels of controls and the commercial plasma were assessed during this step.

Precision and repeatability

The intra-run repeatability was assessed by performing 5 individual measurements of QCs and commercial reference plasma within the same run. The inter-run repeatability was assessed on 10 runs of measurement of the QCs and the commercial reference plasma by the same operator. Reagents were freshly reconstituted or thawed at each run. The intermediate precision was assessed by performing the analyses by 3 different operators. Each operator performed 3 runs measuring the QCs and the commercial reference plasma.

Routine use evaluation

Determination of quality controls and commercial reference plasma values

The QCs and commercial reference plasma values have been determined on 10 different independent runs. Means and standard deviations (SD) enabled the definition of the acceptance range of the 3 levels of controls, which corresponded to the mean ±2*SD. For the commercial reference plasma, the accepted range was defined as the percentage of inhibition that corresponds to 90% ± 2.5% of inhibition of the in-house reference plasma. The 3 levels of QCs and the commercial reference plasma were assessed at each run (n=23 for the first batch of reagent and n=19 for the second batch of reagent).

The sensitivity of the commercial reference plasma was compared to the in-house reference by using the nAPCsr equation referred in **Equation 2**. A "nAPCsr correction factor" was determined by computing the nAPCsr of the commercial reference plasma (numerator) using the in-house reference plasma as the reference plasma (denominator) (**Equation 4**). This "nAPCsr correction factor" was computed by performing 6 independent runs. In addition, an "ETP correction factor" was defined during this step, as the ETP ratio of the commercial reference plasma compared to the in-house reference plasma, both in the absence of APC (**Equation 5**). The "ETP correction factor" ensures the recovery of the true ETP ratio of a sample versus the in-house reference plasma.

Equation 4 :

$$\text{nAPCsr correction factor (F)} = \frac{Commercial\ reference\ \ plasma\ ETP\ (+APC) \Big/ Commercial\ reference\ \ plasma\ ETP\ (-APC)}{In-house\ reference\ \ plasma\ (+APC) \Big/ In-house\ reference\ \ plasma\ (-APC)}$$

Equation 5 :

$$\text{ETP correction factor (CF)} = \frac{Commercial\ reference\ plasma\ ETP\ (-APC)}{In-house\ reference\ plasma\ ETP\ (-APC)}$$

Inter-batch management

Inter-batch management was performed according to the CLSI-EP26-A document. Twelve samples involved in the stability study, the QCs and the commercial reference plasma from the previous batch were tested to ensure recovery between the previous and the new batch of reagents. The acceptability range for each of these samples was assessed using the following formula:

Equation 6 :

Critical analytical difference (CD) = $2.77 \times estimated\ CV$

Equation 7 :

Critical SD = $\frac{CD}{100} \times inhibition\ percentage\ with\ current\ batch$

Equation 8 :

Acceptability range = inhibition % with current batch ± SD critical

It is worth noting that the coefficient of variation (CV) being inversely proportional to the percentage of inhibition, the estimated CV mentioned in **Equation 6** was adapted for each sample regarding its initial percentage of inhibition.

Sample stability during storage

Stability after plasma sample freezing for ETP-based APCR assay

Fresh plasma from the 6 healthy males and 6 women taking hormonal contraceptives were tested at different time points to assess the stability of the ETP-based APCR assay. Tests were performed:

- On fresh samples (the day of plasma collection after centrifugation; day 0)
- On frozen samples (≤ -70°C), at day 1, day 15, month 1, month 3, month 6, month 9, month 12, month 18, month 24 and month 36. Note that, as an evaluation of the same samples was also performed at batch changing (i.e., after 5 months), these results have also been included in this stability study.

The stability of thrombin generation results, once the plasma has been frozen at -70°C or lower, was assessed using the method of the rejection limit (RL). This method aims to establish the length of sample storage time during which the measurement of the ETP-based APCR is acceptable when samples are handled and stored carefully following the laboratory's established conditions.

The impact of freezing was assessed comparing the results of each sample at day 0 (i.e., fresh sample) to the results obtained at day 1.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 8.0 for macOS (GraphPad Software, San Diego, California, USA, www.graphpad.com). Descriptive statistics were used to analyse the data. The variability is expressed in terms of SD instead of CV. Indeed, the expression of the variability in terms of CV is not appropriate in assays with stable SD along the whole range of measurements (Clinical and Laboratory Standards Institute, 2016). For intermediate precision, data were compared using an ANOVA with Tukey's multiple comparison's test. For non-linear regressions, one-phase association regressions were used when linear correlations were impossible. Linear regressions and Pearson correlations were used to assess the correlation between the nAPCsr and the percentage of inhibition. Comparison of results at D0 and D1 for stability testing was performed by a Wilcoxon-signed rank test if distribution failed to show normality using the Shapiro-Wilk normality test. Otherwise, a Mann-Whitney test was used. The different groups of donors were compared using a Kruskal-Wallis test along with the Dunn's test for multiple comparison.

RESULTS

Validation of the ETP-based APCR assay

The results of the method validation are summarized in **Table I.1.1**.

Linearity analysis

The dose-response curve of the APC is shown in **Figure I.1.1(A).** The concentration of APC to obtain a 90% (accepted range: 87.5% to 92.5%) inhibition of the ETP with the two different reference plasmas were 142 (accepted range: 128 to 161) mU/mL and 362 (accepted range: 305 to 485) mU/mL for the in-house plasma and the commercial reference plasma respectively. The dose-response curve of the protein S is shown in **Figure I.1.1(B)**.

Stability of the reagents

Results showed that reagents are stable for 4h after reagent reconstitution and stabilization at room temperature.

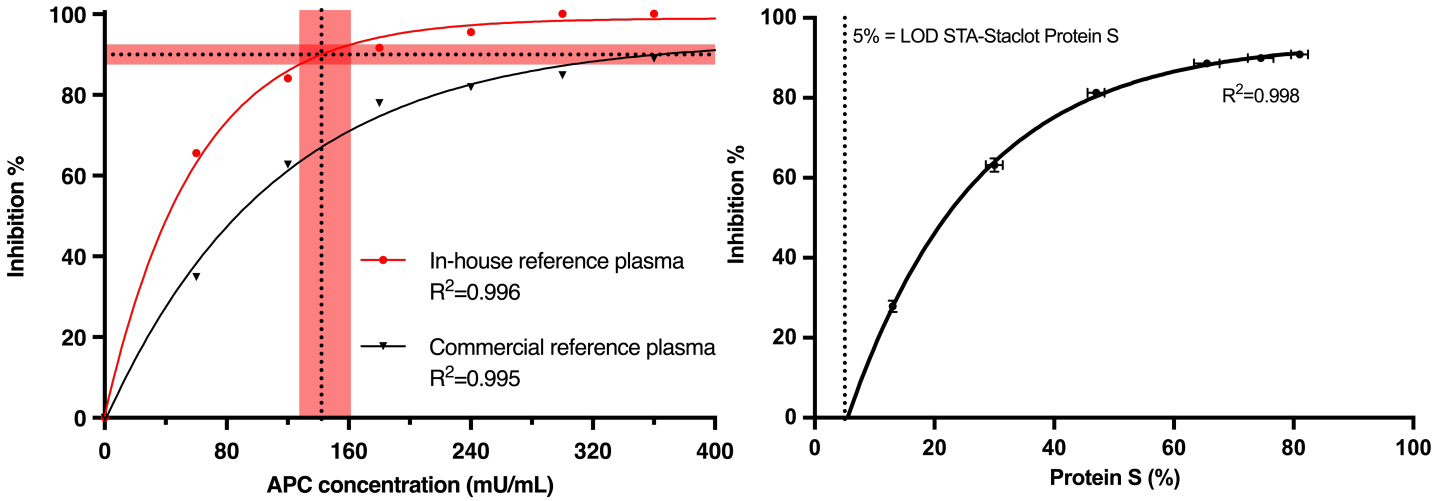


Figure I.1.1 | (A) Inhibition percentage of the ETP at various concentrations of APC with two different reference plasmas. (B) Inhibition percentage depending on protein S deficiency.

Log-transformation of the X-axis did not permit obtaining a linear correlation. A non-linear model was used as recommended (European Medicines Agency, 1995; Food and Drug Administration, 2018). R^2 of the non-linear regressions were both > 0.99. The concentrations of APC required to obtain a 90% (accepted range: 87.5 to 92.5%) of inhibition of the ETP with the in-house reference plasma and with the commercial reference plasma were 142 (accepted range: 128 to 161) mU/mL and 362 (accepted range: 305 to 485) mU/mL. The Y-dotted line and the dashed zone represent 90 ±2.5 % of inhibition. The X-dotted line and the corresponding dashed zone the chosen APC concentration (based on the in-house reference plasma) and its acceptance range. For protein S deficiency, vertical dotted line represents the limit of detection of the STA®-Staclot®Protein S assay (results beyond this limit were not considered)

Precision and repeatability

Intra-run, inter-run repeatability and intermediate precision showed SD values within the acceptance criteria (**Table I.1.1**).

Reference range and sensitivity towards different oral contraceptive therapies and factor V Leiden mutation

In the reference range study involving 47 healthy subjects, the mean percentage of inhibition was 90% and the nAPCsr was 1.04. In the different contraceptive groups, the mean percentage of inhibition and the nAPCsr were 72% and 2.72, 45% and 5.40, 83% and 1.62 for 2nd generation COC, 3rd generation COC and progestin-only contraceptive, respectively (details are provided in **Table I.1.2** and **Figure I.1.2**). Carriers of a heterozygote FVL mutation showed a percentage of inhibition of 35% and 60% which represents a nAPCsr of 5.82 and 4.05, respectively (**Table I.1.2** and **Figure I.1.2**).

Routine use evaluation

Determination of the quality controls and the commercial reference plasma values

Mean percentages of inhibition together with acceptance ranges of QC low, QC intermediate, QC high and the commercial reference plasma after 10 independent runs are summarized in **Table I.1.3**. The mean percentage of inhibition of the commercial reference plasma was 65%. The "nAPCsr correction factor" and the "ETP-correction factor" of the commercial reference plasma were 3.156 (SD = 0.12; n=6) and 1.271 (SD = 0.08; n=6) respectively, compared to the in-house reference plasma.

The acceptance range of the commercial reference plasma corresponding to the acceptance range of the in-house reference plasma (i.e., 87.5% to 92.5%) was 56% to 74%.

Inter-batch variability

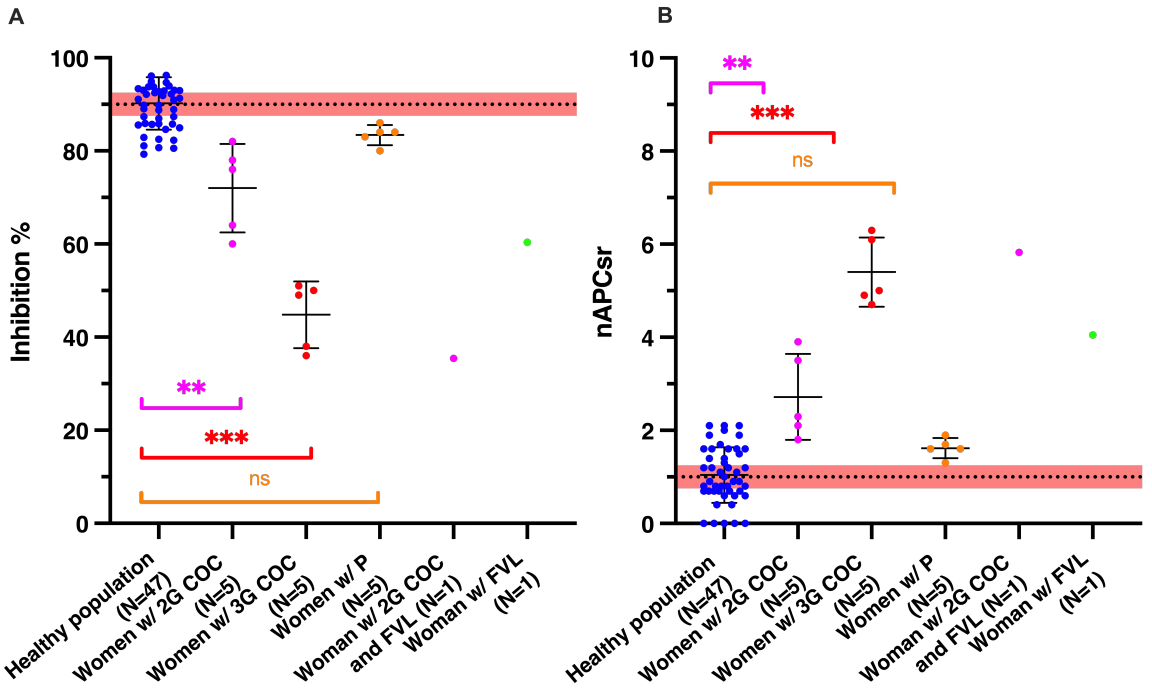
All the 12 samples included for the evaluation of the inter-batch variability as well as the 3 QC levels and the commercial reference plasma were within acceptance ranges (**Table I.1.3**).

The within-batch variability is in line with the between-batch relative differences. The SD was always ≤6% for all QCs over the two batches of reagents (**Table I.1.4**).

Table I.1.2 | Inhibition percentages and nAPCsr values among the 47 healthy subjects and in the 15 women included in the sensitivity study.
Differences between the groups are statistically significant for women on 2nd and 3rd generations COC compared to healthy subjects. No difference was observed for women with progestin only therapy. Results were similar whether expressed as a percentage of inhibition or nAPCsr.

	Percentage of inhibition				nAPCsr				
	Mean	**95% CI**	**SD**	**10th – 90th percentile**	**Mean**	**95% CI**	**SD**	**10th – 90th percentile**	**p-value***
Healthy subjects (n=47)	90%	89 to 92%	6%	82% -100%	1.04	0.87 to 1.21	0.59	0.00 – 1.92	-
Women on 2nd generation COC (n=5)	72%	60% to 84%	9%	60% - 82%	2.72	1.57 to 3.87	0.92	1.80 – 3.90	0.0036
Women on 3rd generation COC (n=5)	45%	36% to 54%	7%	36% - 51%	5.40	4.48 to 6.32	0.74	4.70 – 6.30	0.0002
Women on progestin-only contraceptive (n=5)	83%	81% to 86%	2%	80% - 86%	1.62	1.35 to 1.89	0.22	1.30 – 1.90	0.1948
Woman on 2nd generation COC and FVL heterozygous mutation (n=1)	35%	N.A.	N.A.	N.A.	5.82	N.A.	N.A.	N.A.	N.A.
Woman with FVL heterozygous mutation (n=1)	60%	N.A.	N.A.	N.A.	4.05	N.A.	N.A.	N.A.	N.A.

*p-value was assessed using a Kruskal-Wallis test with Dunn's test multiple comparison and compared healthy subjects with the 3 other groups individually.



The mean percentage of inhibition is higher in the healthy population and in women on progestin-only contraceptive (n=2 for etonogestrel implants, n=2 for oral desogestrel and n=1 for oral nomegestrol). Compared to the healthy population, women on 2nd and 3rd generations COC and carriers of a heterozygote FVL mutation have a lower percentage of inhibition showing a tendency for some resistance towards APC. The Y-dotted lines and the dashed zones represent 90 ±2.5 % of inhibition (on the right) and the corresponding nAPCsr of 1.00 ±0.25 (on the left).

Figure I.1.2 | Percentage of inhibition and corresponding nAPCsr, obtained with the validated ETP-based APCR assay, in a healthy population (men and women not using hormonal therapy), in women on 2nd generation COC, in women on 3rd generation COC, in women on progestin-only contraceptive and in carriers of FVL mutation.

Table I.1.3 | Inter-batch variability and definition of the acceptance range for the 12 random samples, the QCs and the commercial reference plasma included for evaluation of batch changing.

All percentages of inhibition obtained with the second batch of reagents were within acceptance ranges obtained with the first batch of reagent.

Sample ID	Inhibition % (1st batch)	Acceptance ranges	Inhibition % (2nd batch)	Validated if inhibition % new batch within range
QC Low*	100%	100 to 100%	100%	✓
QC Intermediate*	17%	11 to 24%	19%	✓
QC High*	6%	1 to 12%	3%	✓
Reference plasma*	65%	56 to 74%†	65%	✓
Contraceptive 1	67%	63 to 71%	71%	✓
Contraceptive 2	76%	72 to 80%	76%	✓
Contraceptive 3	35%	29 to 42%	33%	✓
Contraceptive 4	43%	37 to 48%	47%	✓
Contraceptive 5	63%	59 to 67%	64%	✓
Contraceptive 6	40%	35 to 46%	46%	✓
Man 1	94%	90 to 98%	97%	✓
Man 2	100%	96 to 100%	97%	✓
Man 3	90%	86 to 94%	91%	✓
Man 4	90%	86 to 94%	90%	✓
Man 5	91%	87 to 95%	93%	✓
Man 6	93%	89 to 97%	94%	✓

*The QCs and the commercial reference plasma were those validated with the first batch of reagents. †The acceptance range of the reference plasma corresponds to the acceptance range of the in-house reference plasma (i.e., 87.5% to 92.5%)

Table I.1.4 | Mean values and standard deviations of the different QCs and the commercial reference plasma over the 2 batches of reagents.

Results are expressed as inhibition percentages. Within-batch and between-batch SD value <5% and 10%, respectively, is considered as a very reproducible method.

	Mean value – SD				Mean SD
	QC Low	QC Intermediate	QC High	Commercial reference plasma	
All (n=41)	100% - 0%	25% - 6%	7% - 2%	67% - 3%	4%
Lot 1 (n=23)	100% - 0%	21% - 3%	7% - 3%	66% - 4%	3%
Lot 2 (n=18)	100% - 0%	31% - 2%	6% - 2%	68% - 3%	2%

Expression of the results – inhibition percentage versus nAPCsr

On the 64 plasma samples from healthy subjects (n=47), women on contraceptives (n=15) or carriers of FVL mutation (n=2), the correlation's Pearson coefficient was -0.998 (95% CI: -0.999 - -0.997) **(Figure I.1.3).**

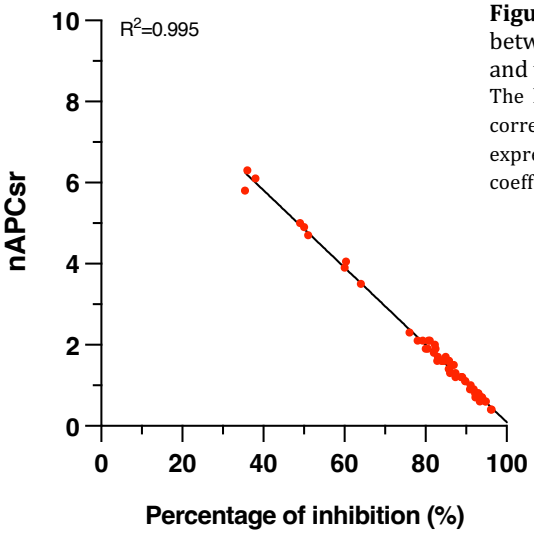


Figure I.1.3 | Linear correlation between the percentage of inhibition and the nAPCsr.
The linear correlation reveals an excellent correlation between the two ways of expression (R^2=0.995). The Pearson r coefficient is -0.998 (95% CI: -0.999 -0.996).

Sample stability during storage

On the 12 donors included in the stability study, Shapiro-Wilk normality test did not reveal a normal distribution. Thus, a Wilcoxon signed rank test was used to assess the difference between the paired two groups. Results showed that the ETP-based APCR is impacted by freezing (p-value = 0.0005). Once frozen, samples are stable for at least 36 months as demonstrated by the rejection limit analysis (**Figure I.1.4**).

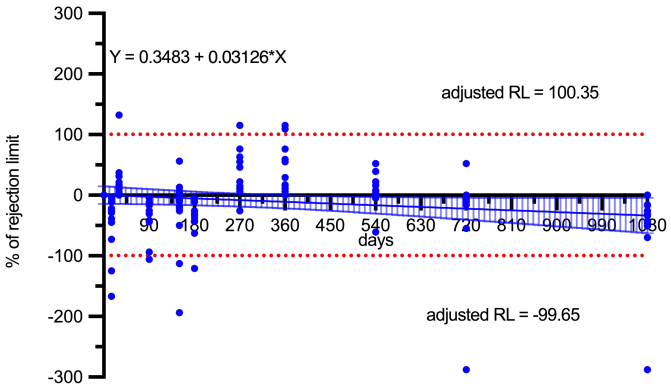


Figure I.1.4 | Stability after sample freezing (<-70°C) of the percentage of inhibition with the ETP-based APCR assay.

The percent bias (or the %RL) was plotted versus time for each time point. The regression line of this plot represents the average percent bias (proportional change) over time. The 100% RL has been defined as the maximal allowable error (MAE). For the percentage of inhibition, the MAE was 20% based on the data from the precision study. The Y-intercept was used to determine the adjusted RL (upper adjusted RL = 100 + Y-intercept – lower adjusted RL = -100 + Y-intercept). The 95%CI of the linear regression (the blue hashed zone) does not cross the adjusted RL which means that the samples are stable over the analysed period (i.e., 1080 days). Samples outside the rejection limit are included in the fitting of the linear regression. They represent less than 10% of the measures.

DISCUSSION

This study is the first reporting a complete validation of the ETP-based APCR assay according to the regulatory requirements (European Medicines Agency, 1995; Food and Drug Administration, 2018). Results showed that the ETP-based APCR test, performed according to this validation, provides reproducible, sensitive, and validated results, building the first steps to implement this assay into routine (**Table I.1.1**). Besides this progress, this will finally allow study-to-study comparison. Indeed, the current methodologies found in the literature are not standardized nor validated and do not usually describe procedures for ensuring reproducibility from batch-to-batch (Curvers, Christella, et al., 2002). In addition, modifications in the concentration of TF, PLs, and exogenous APC made the sensitivity of the test vary (Curvers, Christella, et al., 2002; Lebreton et al., 2017).

Within and between-run reproducibility showed similar SD on the whole range of measurements (**Table I.1.4**) demonstrating a same precision of the assay whatever the level of inhibition of the sample. Intermediate precision showed that, whatever the trained operator, the test provides reproducible results. The daily management of the 3 QC levels provides satisfactory results. They were always within the acceptance range among the 41 different experiments distributed on 2 batches of reagents. Batch-to-batch variation is also efficiently managed with total recovery of the QCs, reference plasma and 12 real-life samples from donors covering the entire range of measurement of the test. Concerning the sensitivity, the assay is able to discriminate healthy subjects from women treated with 2nd or 3rd generation COCs even in this small subset of subjects, i.e., with only 5 subjects in each treated group. In addition, 2 women with FVL mutation were also included in this study. Their nAPCsr values were 4.05 and 5.82, confirming that the test is also sensitive to this genetic mutation. Interestingly, among these 2 subjects, the highest value was obtained in a woman concomitantly treated with a 2nd generation COC, suggesting that the test is also sensitive to the synergistic effect of these two well-known conditions predisposing to APCR.

The dose-response curves demonstrated that the test is sensitive towards levels of protein S in the tested sample as well as from the levels of exogenous APC added in the test, as already reported by others (Curvers, Christella, et al., 2002; de Visser et al., 2005). Results also showed an excellent correlation between the percentage of

inhibition and the nAPCsr, which implies that the use of a reference plasma in each plate to calculate the nAPCsr is maybe no longer needed (**Figure I.1.3**). However, as previously demonstrated by several authors, the use of a reference plasma is needed to reduce the inter-experiment variability of TGT parameters such as the lag time, time-to-peak, peak or ETP. Thus, a strategy to manage the use of a commercial reference plasma is needed, as proposed in this validation (Dargaud et al., 2010; Dargaud et al., 2007; Dargaud et al., 2012; Perrin et al., 2015). Regarding the stability, our results demonstrated an impact of freezing on the ETP-based APCR. However, once frozen, these samples are stable for at least 36 months (**Figure I.1.4**)**.**

This study has limitations. First, this is a monocentric study. Therefore, the inter-laboratory performance of this validated ETP-based APCR assay could not be assessed. Secondly, results of external quality assurance program have not been included in this validation as the current thrombin generation EQA program is not designed for thrombin generation assessment of APCR (Kluft et al., 2010). Finally, the study was not conducted nor powered to define the normal range in patients treated with hormonal therapies or having a FVL mutation. In this first investigation of the analytical performance, only a small subset of samples has been included. The definition of the different normal ranges should be preferably assessed in 120 samples in each group (Clinical and Laboratory Standards Institute, 2010) but this is not the scope of this study since we aimed at proposing a standardized methodology that could be used in many laboratories first. Thus, only trends can be drawn on the overall effect of the different hormonal therapies or FVL mutation and further works are necessary to define the variables that would yield results outside the reference range.

Reference plasma

Previous publications recommended the use of a reference plasma to reduce the inter-experiment and the inter-laboratory variability (Dargaud et al., 2010; Dargaud et al., 2007; Dargaud et al., 2012; Perrin et al., 2015). The use of a commercial reference plasma within this validation agrees with the literature since the use of local plasma (i.e., a reference plasma performed at the laboratory facilities) was generally reported as unable to improve the inter-laboratory variability and even worsening the CV after normalization (Dargaud et al., 2010; Loeffen et al., 2012; Perrin et al., 2015). The choice of a commercially available and certified plasma is therefore more appropriate. On the other hand, a certain heterogeneity between the different commercial reference plasma, which may hinder the benefit of their use, was reported (Dargaud et al., 2010). In addition, the choice of a freeze-dried plasma can be challenged since these plasmas have higher thrombin generation capacity than frozen plasma due to multiple pre-analytical reasons (Dargaud et al., 2010; Rodgers et al., 2014). This is also observed regarding the sensitivity towards

added APC (**Figure I.1.1**). In any case, the conditions behind the large-scale production of these commercial reference plasma are probably far away from the best recommendations of blood samples collection for thrombin generation testing. Thus, one solution may be to compare the response of the commercial reference plasma with a smaller pool of plasma from healthy donors collected in ideal conditions and then to apply a correction factor to the commercial plasma to compensate for their differences. This approach is efficient since the nAPCsr, calculated in this validation for both the healthy subjects and the subjects on hormonal contraceptive, is similarly sensitive to the percentage of inhibition and closely correlates with this parameter (**Figure I.1.3** and **Table I.1.2**).

Quality controls

Interestingly, it has been reported that an internal quality control (IQC) is essential to produce consistent results of thrombin generation (Bagot et al., 2015). However, in the field of thrombin generation, the confusion between an IQC and a reference is often made. Most studies have proposed to normalize the results according to a reference plasma (Bagot et al., 2015; Dargaud et al., 2010; Dargaud et al., 2007; Dargaud et al., 2012; Perrin et al., 2015), which is not the same as a true control plasma. To comply with local legislation, regulations, guidelines, or standards issued by relevant bodies, at least 2 levels of controls are recommended. It is of upmost importance for thrombin generation to propose such different levels of control to allow the implementation of thrombin generation into routine testing. This ensures the technique is under control for both normal and abnormal sample analysis.

Over a series of 41 measurements of the QCs, we demonstrated that the SD of the 3 levels of controls were always ≤3% for each batch taken individually (**Table I.1.4**). These data confirm that this validated ETP-based APCR assay is an accurate thrombin generation application that could be performed routinely to assess patients' prothrombotic state due to resistance towards APC.

Impact of freezing and stability of the samples

Information in the literature is sparse regarding the stability of plasma samples for thrombin generation testing. A previous study evaluating the impact of pre-analytical parameters on the measurement of circulating microparticles with thrombin generation revealed that plasma samples are stable for one year at -80°C (Lacroix et al., 2012). In this previous study, the authors assessed the stability of thrombin generation with the MP reagent from Thrombinoscope BV. This reagent does not contain TF as opposed to the trigger reagent assessed in this study (concentration around 5pM). This higher TF concentration provides robustness to the analytical procedure. However, we showed that freezing impacts the ETP-based

APCR but once frozen, plasma samples are stable during at least 36 months (**Figure I.1.4**).

Normal range definition

In this study, 47 samples from healthy donors were tested to provide grounds for the definition of a normal range for this ETP-based APCR assay and also to validate the use of a correction factor on the commercial reference plasma to match residual activity. The mean percentage of inhibition, i.e., 90%, and the mean nAPCsr, i.e., 1.04, demonstrate that the concentration of APC used to obtain 90% of inhibition of the ETP in our in-house reference plasma is correct. When compared to the results obtained in subjects treated with hormonal contraceptive therapy, the 10^{th}-90^{th} percentile range (**Table I.1.2**) could serve as a basis for the classification of normal and abnormal plasma. It could be proposed that samples above the 90^{th} percentile (for both the percentage of inhibition and the nAPCsr) may represent samples in which resistance to APC is suspected. However, further studies in larger cohorts of subjects on hormonal therapies, during pregnancy, with FVL or PT G20210A mutations and with clinical thrombotic events are needed before adopting this assumption.

CONCLUSIONS

This is the first study presenting a validated ETP-based APCR assay with performance data. Results revealed an excellent inter-experiment precision thanks to the standardized methodology, the excellent reproducibility and the use of a commercial reference plasma and quality controls to validate each run of measurements. This validation provides pharmaceutical industries, regulatory bodies and physicians with a reproducible, sensitive, and validated assay that could be proposed as a gold standard for the assessment of the ETP-based APCR. This finally ensures study-to-study comparison as well as perspectives for the establishment of specific thresholds to reflect the prothrombotic state in individual patients. Multicentric studies and external quality assurance programs are required to assess the inter-laboratory reproducibility of this technique and confirm its usefulness for standardizing thrombin generation testing.

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I.2 - INTERLABORATORY VARIABILITY ASSESSMENT OF APC RESISTANCE USING THE ETP-BASED APC RESISTANCE ASSAY

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SUMMARY

Background and objectives: Although the endogenous thrombin potential-based activated protein C resistance (ETP-based APCR) is recommended for the development of steroid contraceptive agents, one of the main limitations of this technique was its lack of standardization, which hampered study-to-study comparison. A validated methodology that met all the regulatory requirements in terms of analytical performances has been developed recently. To ensure a wide implementation of this test, the assessment of the interlaboratory variability was needed.

Method: The assay was implemented in three testing laboratories**.** First, dose-response curves were performed to locally define APC concentration leading to 90% of ETP inhibition on healthy donors. Intra- and inter-run repeatability were assessed on a reference plasma and three quality controls. To investigate the variability in results among the different testing units, 60 donor samples were analysed at each site.

Results: The APC concentration leading to 90% of ETP inhibition was defined at 1.21 µg/mL and 1.14 µg/mL in the two receiving units. Intra- and inter-run repeatability showed standard deviation below 3%. Analyses of the 60 donor samples showed no statistically significant difference. The sensitivity of the test in the different laboratories was maintained and subgroup analyses still reported significant differences depending on hormonal status of donors.

Conclusion: This study is the first reporting the interlaboratory variability of the ETP-based APCR assay. Data revealed excellent intra-laboratory precision and interlaboratory reproducibility. These results support the concept that this blood coagulation test provides an appropriate sensitivity irrespective of the laboratory in which analyses are performed.

BACKGROUND

Evidence suggesting that combined oral contraceptives (COCs) were associated with an increased risk of venous thromboembolism (VTE) rapidly appeared after their marketing in the 1960s (Jordan et al., 1961). Subsequently, numerous studies assessing the impact of the different estroprogestative combinations on haemostasis demonstrated that a poor response to the activated protein C (APC) could explain, at least in part, the procoagulant state observed in users of oral contraceptive agents (Henkens et al., 1995; Olivieri et al., 1995; Osterud et al., 1994; Rosing et al., 1997). Several methods were therefore developed to evaluate this acquired APCR among which the endogenous thrombin potential (ETP)-based APCR assay. This test is based on the continuous measurement of thrombin generation, in the presence and absence of exogenous APC (Nicolaes et al., 1997). As a global coagulation assay, it takes into account the initiation, the propagation and the termination phase of the coagulation providing additional information, notably on the prothrombin (PT) G20210A mutation, antithrombin and protein S deficiencies or FVIII levels (Duchemin et al., 2008; Lavigne-Lissalde et al., 2010; Marco et al., 2012). In 2005, this blood coagulation test was recommended by the European Medicines Agency (EMA) for the evaluation of APCR during the development of steroid contraceptive agents in women (European Medicines Agency, 2005). However, a major limitation of this technique was its lack of standardization, which hampered study-to-study comparison (Dargaud et al., 2012; Perrin et al., 2015). Hence, our group recently proposed a standardized methodology that met all the regulatory requirements in terms of analytical performances. As described in **Part I.1**, the ETP-based APCR assay was validated on the Calibrated Automated Thrombogram (CAT) device using commercially available reagents to ensure batch-to-batch traceability, recovery and reproducibility of the method over time (Douxfils, Morimont, Delvigne, et al., 2020). The transfer of this methodology to different laboratories is a next step for the wide implementation of this promising test in the routine laboratory setting.

The aim of this study was to demonstrate the robustness of the method through an evaluation of the interlaboratory transferability, ensuring that any laboratory implementing the method obtains similar results in its environment compared to those obtained by the originating laboratory. To this end, the ETP-based APCR assay was implemented in 2 different laboratories and results were compared to those obtained by the reference laboratory.

MATERIAL AND METHOD

ETP-based APCR assay

The ETP-based APCR assay was performed as described in **Part I.1**. and illustrated in the **Memento**. Briefly, the activator reagent (STG® ThromboScreen -TM) (TS) contains a mixture of phospholipids (PLs) (±4µM) and tissue factor (TF) (±5pM) to initiate coagulation through the extrinsic pathway in the tested plasma. The addition of exogenous APC (Enzyme Research Laboratories, Swansea, UK) to this reagent enhances the APC – protein S anticoagulant complex in the tested plasma. The resulting effect is a reduction of the ETP (corresponding to the area under the curve) in comparison with non-APC condition. The quantity of APC to introduce in the test is defined per APC/TS batch and targets a decrease of 90% of the ETP of a reference plasma.

The ETP values measured with TS (-APC) and TS (+APC) are then used to compute the percentage of inhibition of the ETP (inhibition %) (**Equation 3**), and the normalized APC sensitivity ratio (nAPCsr) (**Equation 2**). The obtained ratio stands between 0 and 10 and the higher the nAPCsr, the more resistant the donor is to APC.

Reference plasma

As already explained in **Part I.1**, the use of a reference plasma is dedicated to the normalization of thrombin generation test (TGT) parameters. It also determines the quantity of APC to introduce in the test, to obtain 90% of ETP inhibition. In our study, we used 2 different reference plasmas: an in-house healthy pooled plasma (HPP) and a commercially available reference plasma (STG-RefPlasma TS). The pros and cons of using one or the other have been discussed in the method validation.

Quality controls

Three levels of quality controls (QC) were used to validate each experiment. These are the same as those used for the validation, i.e., QC low (STG-QualiTest Low TS), QC intermediate (STG-QualiTest Norm TS) and QC high (STG QualiTest High TS). Acceptability ranges of these controls are based on 10 consecutive runs and are defined as the mean ± 2*standard deviations (SD). These acceptability ranges were defined for each batch by the originating unit. However, as the residual ETP of QC low in presence of APC is usually zero, this results in 100% inhibition and thus no range for this QC level can be determined. If this QC level is slightly below 100% inhibition, but the other QC levels and the reference plasma are within their acceptability ranges, the run is validated.

Method transferability

The transferability of the methodology was investigated at the department of pharmacy of the University of Namur (Namur, Belgium) (named **receiving unit 1**) and at the haematology laboratory of Centre Hospitalier Universitaire (CHU) Estaing (Clermont-Ferrand, France) (named **receiving unit 2**). The originating unit, i.e., QUALIblood sa (Namur, Belgium) was responsible for providing the analytical procedures, reagents, and samples to the receiving laboratories. One operator at each site, already familiar with TGT, has been trained remotely before the transfer.

The transferability assessment was performed in 3 steps (**Figure I.2.1**): [(1)]the determination of the APC concentration at each site, [(2)]the assessment of the method precision, [(3)]the assessment of the interlaboratory variability.

Figure I.2.1 | Study scheme for the transferability of the ETP-based APCR assay

Step 1: Determination of APC concentration

The first step consisted of defining the amount of exogenous APC to be added in the TS reagent for the +APC condition. To this end, dose-response curves were performed with 6 solutions of TS containing increasing concentrations of APC: 0.5µg/mL; 0.75µg/mL; 1.25µg/mL; 2. µg/mL; 2.75µg/mL and 3.5µg/mL. The chosen APC concentration was the one leading to a target inhibition % of the commercial reference plasma defined by the originating unit. For the receiving unit 1, the targeted inhibition of the reference plasma (batch 202983) was 57.8% and for the receiving unit 2, the targeted inhibition % of the reference plasma (batch 202984) was 75.0%. To ensure this concentration was properly determined, an additional verification step has been set up and dose-response curves were performed in

parallel on the HPP. The inhibition % of the HPP should stand between 87.5% and 92.5%.

Step 2: Assessment of precision

After defining the right concentration of APC to introduce in the test, receiving units had to be able to replicate with an acceptable level of performance the methodology. To this end, the precision, i.e., the intra- and inter-run repeatability, was assessed. The intra-run repeatability was assessed by analysing the 3 QC levels and the reference plasma on the same plate, 5 times in duplicates. For the inter-run repeatability, those plasmas were analysed in duplicate over 3 independent runs. Results were expressed as inhibition % and the acceptance criteria were the same as for the method validation, i.e., SD values lower than 10%.

Step 3: Assessment of the interlaboratory variability

This step consisted of investigating the variability in results of donor samples among the originating unit and both receiving units. Sixty individual plasma samples were analysed at each site. A comparison between 4 subgroups according to sex and the use of hormonal contraception in women was performed as these parameters are known to impact the resistance toward the APC. Results were expressed as nAPCsr values.

Sample description

Healthy pooled plasma (HPP)

This pool was used to confirm that the chosen APC concentration, in both receiving units, led to 90% ±2.5% inhibition. This in-house HPP was composed of 20 healthy individuals (10 men and 10 women not taking contraceptive agents). Exclusion criteria were history of thrombotic and/or haemorrhagic events, treatment by antiplatelets or anticoagulant medications or other drugs potentially affecting platelets or coagulation, pregnancy, use of hormonal therapy (i.e., contraceptive or hormone replacement therapy), and carrier of factor V Leiden (FVL) or prothrombin (PT) G20210A mutations. The absence of FVL and PT G20210A mutations was confirmed by a CE-IVD-approved technique (LaCAR MDx Technologies S.A., Liège, Belgium). The mean age of the participants was 25 years (ranges, 18-56 years) and the mean body mass index (BMI) was 22 $kg.m^{-2}$ (ranges, 19-26 $kg.m^{-2}$).

Individual plasma

To assess the interlaboratory variability, 60 healthy donors were recruited and stratified in 4 groups of 15 individuals, namely men [mean age, 21 years; mean BMI, 22 $kg.m^{-2}$], women not using hormonal therapy [mean age, 21 years; mean BMI, 22 $kg.m^{-2}$], women using 2^{nd} generation COCs (i.e., containing levonorgestrel as

progestin) [mean age, 22 years; mean BMI, 23 kg.m^{-2}], and women using 3rd generation COCs (i.e., containing desogestrel or gestodene as progestin) [mean age, 23 years; mean BMI, 22 kg.m^{-2}]. The demographic characteristics (age and BMI) of the four groups were well matched. Exclusion criteria were the same as the above-mentioned except for the use of hormonal therapy.

Blood samples collection and plasma preparation

Biological samples were collected in accordance with the Declaration of Helsinki after approval by the Ethical Committee of the Centre Hospitalier Universitaire (CHU), Université Catholique de Louvain (UCL) Namur (Yvoir, Belgium) under the approval number B03920096633. All the volunteers were recruited at the University of Namur, Namur, Belgium. Written informed consent was obtained from each donor. All samples were stored and managed by the Namur Biobank-eXchange (NAB-X), the registered biobank from the University of Namur.

Blood was taken by venepuncture in the antecubital vein and collected into 0.109 M sodium citrate tubes (9:1 v/v) (Vacuette Greiner) without corn trypsin inhibitor using a 21-gauge needle (BD Vacutainer Eclipse). The first tube was always discarded. The platelet poor plasma (PPP) was obtained from the supernatant fraction of blood tubes after double centrifugation for 15 minutes at 2,500 × *g* at room temperature. The first centrifugation was performed within 30 minutes after blood sampling. Immediately after centrifugation, PPP were pooled for the constitution of the HPP or aliquoted as individual plasmas. The aliquots were snap-frozen in liquid nitrogen within 4 hours after the sampling and stored at ≤−70°C. Frozen plasma samples were thawed in a water bath at 37°C for a maximum of 10 minutes and mixed gently just before the experiment. All tests were performed within 4 hours after thawing.

Statistical analysis

Statistical analysis was performed using GraphPad version 9.0 (GraphPad Prism Software, Inc.). Descriptive statistics were used to analyse the data. APC concentrations were determined using non-linear regressions (one-phase association – least square fit) with no constrain on the Y-axis. As the SD is stable along the whole range of measurements, the intra- and inter-run repeatability was expressed in terms of SD instead of the coefficient of variation (CV). A pairwise multiple comparison Friedman test was performed to compare nAPCsr values from each donor between units. Linear regressions and Spearman's correlations were used to assess the correlation between the originating unit versus receiving units. Derived Bland-Altman analysis was performed by plotting differences (nAPCsr units – mean nAPCsr) against mean nAPCsr. Differences were expressed in absolute values as well as percentages of mean results. Within each subgroup, ordinary one-way ANOVAs were performed to compare nAPCsr values between units. Finally, grouped

analysis, including all nAPCsr values obtained at the three units, was performed to compare subgroups with each other.

RESULTS

Determination of APC concentration

Dose-response curves of APC performed at both receiving units are shown in **Figure I.2.2.**

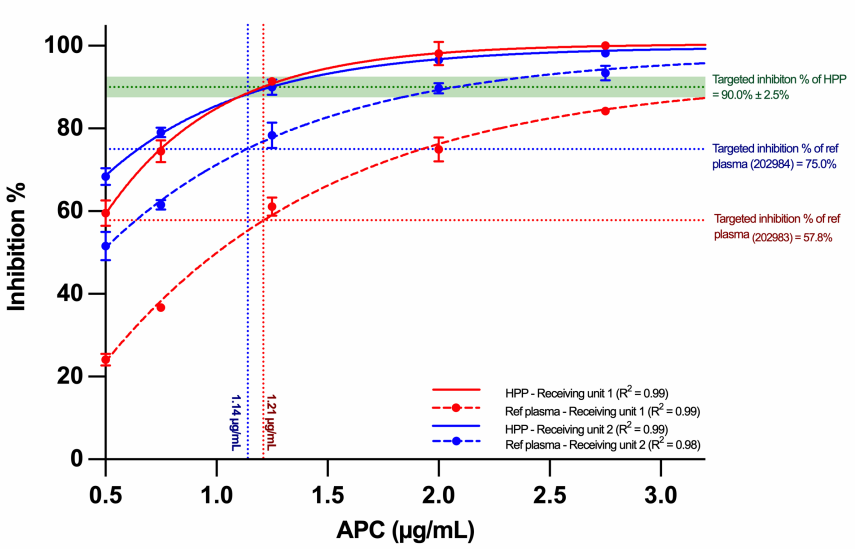

Figure I.2.2 | Inhibition percentage of the ETP at various APC concentrations with the healthy pooled plasma (HPP) and the commercial reference plasma (N=3).
The continuous lines represent the non-linear regressions obtained with HPP. The dotted lines represent the non-linear regressions obtained with the commercial ref plasma. The red lines refer to the receiving unit 1 and the blue lines refers to the receiving unit 2.

Coefficients of determination (R^2) of individuals and mean non-linear regressions for HPP and for reference plasma were ≥0.98 in both receiving units. The concentration of APC leading to 57.8% inhibition (receiving unit 1) and 75.0% inhibition (receiving unite 2) of the reference plasma was 1.21µg/mL and 1.14µg/mL respectively. These concentrations led to an inhibition % of the HPP within the established ranges [87.5%-92.5%], i.e., 90.1% inhibition at receiving unit 1 and 88.6% inhibition at receiving unit 2.

Assessment of method precision

Intra-run and inter-run repeatability at both receiving units are shown in **Table I.2.1** and **Table I.2.2**, respectively. In both units, SD values for each tested plasma (i.e., the 3 QC levels and the reference plasma) were within acceptance criteria (SD <10%). In addition, values were within QC ranges defined by the originating unit except for QC low, at the receiving unit 2, which was below 100% inhibition for 2 runs.

Table I.2.1 | Intra-run repeatability of the commercial reference plasma and QCs (i.e., QC low, QC intermediate and QC high) for each receiving unit.

Tested plasmas	Duplicate	Receiving unit 1				Receiving unit 2			
		Inhibition %	Mean inhibition %	SD	QC ranges	Inhibition %	Mean inhibition %	SD	QC ranges
Reference plasma	1	79.1%	78.8%	0.4%	[70%-82%]	76.5%	75.3%	1.2%	[67%-80%]
	2	78.6%				73.4%			
	3	78.2%				75.2%			
	4	79.1%				75.0%			
	5	79.1%				76.2%			
QC Low	1	100.0%	100.0%	0.0%	[100%-100%]	100.0%	100.0%	0.0%	[100%-100%]
	2	100.0%				100.0%			
	3	100.0%				100.0%			
	4	100.0%				100.0%			
	5	100.0%				100.0%			
QC intermediate	1	39.8%	40.2%	0.7%	[33%-44%]	39.1%	37.7%	0.9%	[24%-53%]
	2	40.4%				37.6%			
	3	41.1%				38.2%			
	4	39.3%				36.8%			
	5	40.3%				37.0%			

Table I.2.1 (continued) | Intra-run repeatability of the commercial reference plasma and QCs (i.e., QC low, QC intermediate and QC high) for each receiving unit.

QC high	**1**	**2.9%**	**2.8%**	**0.7%**	**[0%-17%]**	**2.9%**	**4.0%**	**1.5%**	**[2%-13%]**
	2	2.2%				4.4%			
	3	2.4%				2.1%			
	4	4.0%				4.3%			
	5	2.6%				6.1%			

Table I.2.2 | Inter-run repeatability of the reference plasma and QCs (i.e., QC low, QC intermediate and QC high for each receiving unit).

Tested plasmas	**Duplicate**	**Receiving unit 1**				**Receiving unit 2**			
		Inhibition %	**Mean Inhibition %**	**SD**	**QC ranges**	**Inhibition %**	**Mean Inhibition %**	**SD**	**QC ranges**
Reference plasma	1	78.8%	80.9%	1.9%	[70%-82%]	75.2%	78.5%	1.2%	[67%-80%]
	2	81.5%				79.3%			
	3	82.4%				77.6%			
QC Low	1	100.0%	100.0%	0.0%	[100%-100%]	100.0%	97.9%	1.9%	[100%-100%]
	2	100.0%				97.1%			
	3	100.0%				96.5%			
QC intermediate	1	40.2%	42.8%	2.6%	[33%-44%]	37.7%	39.6%	2.3%	[24%-53%]
	2	42.9%				39.0%			
	3	45.4%				42.2%			
QC high	1	2.8%	5.0%	2.3%	[0%-17%]	4.0%	6.0%	2.1%	[2%-13%]
	2	7.4%				8.1%			
	3	4.9%				6.0%			

Interlaboratory variability assessment

Sixty samples were tested at each unit and compared to each other. However, one sample could not be analysed at the receiving unit 2 due to insufficient volume. A nonparametric Friedman test was performed and revealed no statistically significant differences between units (P>.05). The Spearman correlation depicted in **Figure I.2.3**, showed a significant effective pairing between nAPCsr values from each receiving unit and nAPCsr values from the originating unit. The correlation coefficients (rs) were 0.99 (95% CI, 0.9812 to 0.9935; P<.0001) and 0.99 (95%CI, 0.9851 to 0.9949; P<.0001) for receiving unit 1 and receiving unit 2 respectively. Linear regressions showed the following equations Y = 1.154x-0.2408 (R^2=0.98) for receiving unit 1 and Y = 0.9213x+0.08939 (R^2=0.98) for receiving unit 2.

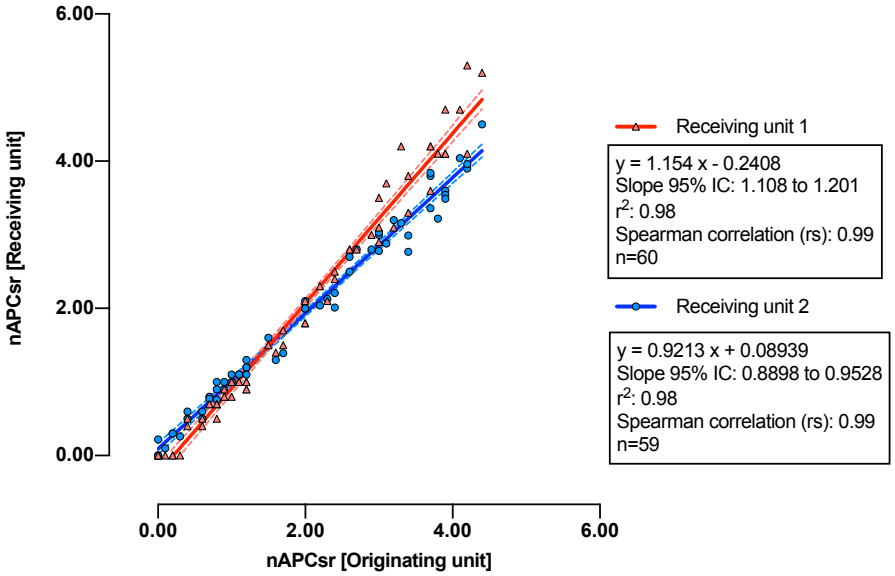

Figure I.2.3 | Correlation between nAPCsr obtained at the receiving units and nAPCsr obtained at the originating unit.

Differences (nAPCsr values from each laboratory – mean nAPCsr from the 3 units), either expressed in absolute values or as percentages of the mean nAPCsr, were plotted against the mean nAPCsr (**Figure I.2.4**).

The average of the differences ±95% confidence interval (CI) for the originating unit equalled 0.00 ± 0.22 in absolute values and 1.3% ± 11.1% in percentages of the mean. The average of the differences ±95%CI for the receiving unit 1 equalled 0.09 ±0.48 in absolute values and -0.5% ±20.5% in percentages of the mean. The average of the differences ± 95%CI for the receiving unit 2 equalled -0.09 ±0.38 in absolute values and -0.8% ±16.8% in percentages of the mean. The 95th percent limits of agreement

of differences, when gathering the three laboratories, equalled 0.40 in absolute values and 16.5% in percentages of the mean.

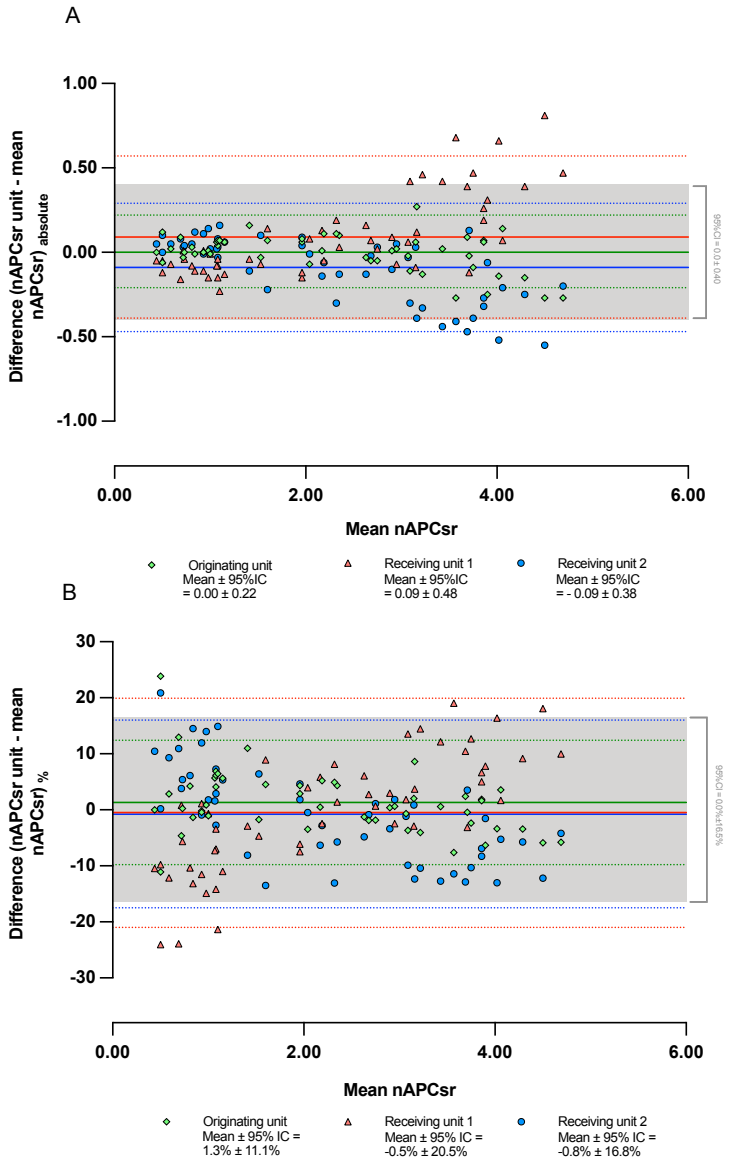

Figure I.2.4 | Derived Bland-Altman analysis between nAPCsr values from each unit and the mean nAPCsr of the three units for the measurement of APC resistance in plasma samples. Differences (nAPCsr unit - mean nAPCsr) are expressed in (A) absolute values or (B) as a percentage of the mean.

Results expressed in percent are computed as follows: $\frac{nAPCsr\ unit - mean\ nAPCsr}{mean\ nAPCsr} * 100$. The originating unit appears in green, the receiving unit 1 in red and the receiving unit 2 in blue.

Comparisons between each unit within the 4 subgroups, i.e., women not using COC, men, women using 2nd generation COCs and women using 3rd generation COCs are shown in **Table I.2.3** and **Figure I.2.5.** Holm-Sidak's multiple comparison tests revealed no significant difference between the 3 laboratories within the 4 subgroups (P>.05). On the other hand, the Tukey's multiple comparisons test showed significant differences between each subgroup (P<.0001 except for comparison between women using 2nd versus 3rd generation COC, in which p-value equalled .0008).

Table I.2.3 | Mean nAPCsr values ± SD of each subgroup obtained at the different units and mean nAPCsr value of each subgroup, regardless of the laboratory unit.

	nAPCsr ±SD			
	Originating unit	**Receiving unit 1**	**Receiving unit 2**	**Mean ± SD**
Women without COC (n=15)	1.19 ±0.42	1.07 ±0.38	1.20 ±0.40	1.15 ±0.40
Men (n=15)	0.49 ±0.47	0.39 ±0.49	0.51 ±0.41	0.46 ±0.45
Women using 2nd COCs (n=15) *	2.87 ±0.86	3.02 ±1.09	2.86 ±0.79	2.92 ±0.90
Women using 3rd COCs (n=15)	3.39 ±0.70	3.71 ±0.94	3.17 ±0.68	3.42 ±0.80

** n=14 for receiving unit 2*

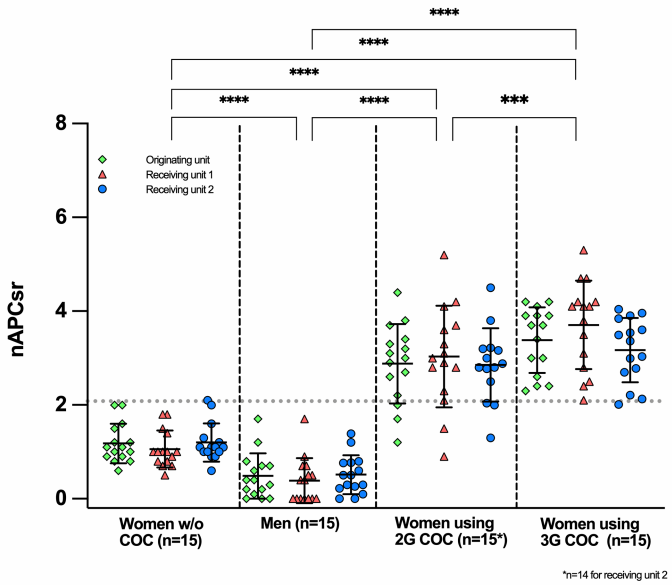

Figure I.2.5 | nAPCsr values of individuals from each subgroup obtained at the different units.

Data obtained at the originating unit figure in green, data obtained at the receiving unit 1 figure in red and data obtained at the receiving unit 2 figure in blue. Means ± standard deviations (SD) are represented. Holm-Sidak's multiple comparison tests were performed to assess the difference between units within each subgroup. Differences between subgroups were assessed by a Tukey's multiple comparison test. Only significant results are shown. P-value format is characterized as follows: * = P ≤0.05; ** = P ≤0.01; *** = P ≤0.001; **** = P ≤0.0001). The gray dotted line represents the upper limit of reference ranges and equals 2.08.

DISCUSSION

The aim of this study was to implement the ETP-based APCR assay in different laboratories and to assess the interlaboratory variability. Data showed that this test, when performed at different facilities, provides reproducible and sensitive results (e.g., depending on hormonal status of women), building an additional step for its implementation in clinical routine.

The critical step, in this study, was the determination of APC concentration. The amount of exogenous APC added into the TS reagent plays a major role in the sensitivity of the test and must be defined at each batch change of APC and/or TS reagent. Indeed, the activity of APC differs from one production to another, as well as TS reagent and the associated reference plasma and quality controls (part of the same commercial kit, STG-ThromboScreen®). For instance, the commercial reference plasma [batch 202983] was more APC-resistant compared to the reference plasma [batch 202984] and the targeted inhibition % of the reference plasma equivalent to 90% inhibition of HPP were 57.8% and 75.0%, respectively. In addition, as the reagent TS +APC is not yet ready to use and still requires the spiking of APC into the TS reagent, an inter-operator variability is non-negligible, especially, as the operators of both external units undergone a distance training, based on the documentation provided by the reference laboratory. The different APC concentrations, i.e., 1.21 µg/mL at receiving unit 1 and 1.14 µg/mL at receiving unit 2, did not seem to impact final results. Indeed, inhibition % of QC levels and the reference plasma, among the receiving units, were within the acceptability ranges defined by the originating unit except for QC low at the receiving unit 2, which was slightly below 100% inhibition compared to the reference unit. As there is no proper range for this control based on our calculation method for the definition of the acceptance range, we accepted these QC values as all other levels of controls were adequate.

Regarding the precision, the within- and between-run variability showed maximal SD of 1.5% and 2.6% respectively, well below the maximal tolerable limit of 10%. These results are in line with those obtained at the reference laboratory (during the validation of the methodology), in which a same degree of precision of the assay was obtained, irrespective of the inhibition level (**Part I.1**).

Finally, to evaluate the interlaboratory variability, results of donors' samples were compared in nAPCsr values rather than inhibition %. As previously mentioned, the nAPCsr is normalized to the reference plasma, the latter being measured at each run, which allows reducing the interlaboratory variability (Douxfils, Morimont, Delvigne, et al., 2020). As expected, Spearman correlations between nAPCsr values obtained at the receiving units versus at the originating unit showed excellent coefficients of

0.98. Based on linear regressions, the receiving unit 1 tended to provide higher nAPCsr values compared to the originating unit with a slope of 1.15, denoting a 15% upward systematic deviation. On the other hand, the receiving unit 2 tended to give lower nAPCsr values compared to the originating unit with a slope of 0.92 corresponding to a downward systematic deviation of 8%. The derived Bland & Altman analysis was performed to compare differences of nAPCsr values from each laboratory and the mean nAPCsr values from the 3 units. The average difference of nAPCsr for each laboratory compared to the mean of the 3 units was very close to zero. In addition, compared to the mean, 95% of nAPCsr values obtained in each laboratory did not differ by more than 0.4 units in absolute values or 16.5% in percent. These results demonstrate the low variability among the three units. Furthermore, subgroup analysis confirmed that the chosen APC concentration in each laboratory, allowed differentiating between men and women not taking hormonal therapy and women using hormonal contraception. Indeed, the reference range based on 47 individuals (men and women not taking hormonal contraception) established during the validation was between 0 and 2.08 (Douxfils, Morimont, Delvigne, et al., 2020). Regardless of the laboratory, mean nAPCsr for women without COC and men's groups were below 2.08 and above 2.08 for women using 2^{nd} and 3^{rd} generation COCs. In addition, no statistically significant differences between the 3 laboratories for each subgroup were observed which means that the methodology remains reproducible, regardless of the measurement range. Finally, pooled data maintained the expected differences between subgroup's, i.e., significant differences between healthy individuals, either men or women not taking COC and women using COCs.

LIMITATIONS AND PERSPECTIVES

One limitation of our study concerns the restricted number of laboratories included due to an insufficient number of aliquots for each sample. However, the between laboratory reproducibility of the method was excellent, despite the remote training of operators (i.e., instead of on-site training). From a technical point of view, there are also several limitations including the turnaround time and the absence of ready-to-use reagents. Indeed, the assay is time-consuming due to reagent preparation, stabilization time, and manual filling of the plate. Furthermore, additional experiments are required at each batch change to define APC concentration and to determine QC ranges. As a perspective, it is therefore of importance to consider the implementation of this assay on a routine clinical equipment and the manufacturing of ready-to-use triggering reagent with APC to throw off these technical restrictions.

CONCLUSION

This study is the first reporting the interlaboratory variability of the validated ETP-based APCR assay. Data revealed excellent intra-laboratory precision and interlaboratory reproducibility. These results support the concept that the nAPCsr, obtained with a validated and standardized methodology, provides an appropriate sensitivity irrespective of the laboratory in which the analysis is performed. This suggests that the nAPCsr could become a promising regulatory and clinical tool to identify the thrombogenicity of COC. Considering its screening potential, the next step is therefore to implement the ETP-based APCR assay in clinical routine.

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I.3 - ANALYTICAL PERFORMANCES OF THE ETP-BASED APC RESISTANCE ASSAY ON THE AUTOMATED ST GENESIA SYSTEM

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SUMMARY

Background: The evaluation of activated protein C (APC) resistance based on the endogenous thrombin potential (ETP) is recommended during the development of steroid contraceptives in women. In 2019, this assay was validated on the Calibrated Automated Thrombogram (CAT) device. However, in view of its screening potential, its automation is essential.

Aims: To transfer the ETP-based APC resistance (APCR) assay on the ST Genesia system using reagent STG-ThromboScreen -TM with exogenous APC added.

Method: Dose-response curves were performed to define APC concentration leading to 90% ETP inhibition on healthy donors. Intra- and inter-run reproducibility was assessed. The normal range was defined based on 56 samples from healthy individuals. The sensitivity was assessed on 40 samples from women using combined oral contraceptives (COCs). A comparison of the method with the validated ETP-based APCR on the CAT system was performed. Results were expressed in normalized APC sensitivity ratio (nAPCsr).

Results: The APC concentration leading to 90% ETP inhibition was 652 mU/mL. Intra- and inter-run reproducibility showed standard deviation below 4%. The nAPCsr normal range stood between 0.00 and 2.20. Analyses of 40 samples from women using COCs confirmed the good sensitivity of the assay. Compared to the CAT system, nAPCsr data were slightly higher on the automated system.

Conclusion: This study is the first reporting the analytical performances of the ETP-based APCR assay on an automated platform. Results support the concept that this test, when performed into clinical routine, could become a promising regulatory and clinical tool to document on the thrombogenicity of female hormonal therapies.

BACKGROUND

Throughout the course of their lifetime, women are exposed to hormonal changes, thus modulating the risk of venous thromboembolic events (VTE). These hormonal changes are either endogenous, as during pregnancy and in the postpartum period or due to the intake of exogenous hormones, as with the use of combined hormonal contraceptives (CHCs) or menopausal replacement therapies (MRTs) during early menopause.

As already discussed, numerous studies have assessed the impact of CHCs and MRT on haemostasis and shown that a poor response to the activated protein C (APC) could explain, at least in part, the procoagulant state observed in women using hormonal therapies, e.g., CHCs or MRTs (Henkens et al., 1995; Olivieri et al., 1995; Osterud et al., 1994; Rosing et al., 1997). In 2005, the European Medicines Agency (EMA) recommended the evaluation of APC resistance (APCR) during the development of steroid contraceptive agents in women (European Medicines Agency, 2005). Initially, the main purpose of APCR testing was to detect the presence of a FV Leiden (FVL) mutation (Kadauke et al., 2014). The original test proposed a ratio between a baseline activated partial thromboplastin time (aPTT) and the aPTT after exogenous APC has been added. However, many interferences on the aPTT assay could falsely lead to APCR. Consequently, over the years, this coagulation test evolved with the aim of making it less or even not sensitive to interferences, e.g., the use of CHCs (Kadauke et al., 2014). Nowadays, the measurement of APCR in women on hormonal therapies should thus rely on the endogenous thrombin potential (ETP)-based APCR assay (Curvers et al., 1999; de Visser et al., 2005). This test was developed more than 20 years ago by Nicolaes *et al.* (Nicolaes et al., 1997) but due to the lack of standardization and harmonization, results were laboratory-dependent hampering study-to-study comparison (Dargaud et al., 2012; Perrin et al., 2015). In 2019, in the light of its potential as a global biomarker of thrombogenicity, our group proposed a standardized methodology to perform the assay, that met all the regulatory requirements in terms of analytical performances (**Part I.1**). The ETP-based APCR assay was validated on the Calibrated Automated Thrombogram™ (CAT) device using commercially available reagents to ensure batch-to-batch traceability, recovery and reproducibility of the method over time (Douxfils, Morimont, Delvigne, et al., 2020). Recently, the inter-laboratory variability study supported the concept that the ETP-based APCR, performed with our validated and standardized methodology, provides an adequate sensitivity and an excellent inter-laboratory reproducibility (**Part I.2**). Nevertheless, the CAT mostly remains a research platform and the implementation of the ETP-based APCR on an automated analyser is of paramount importance to have this innovative biomarker available for the clinicians in their daily practice.

The aim of this study was to implement the ETP-based APCR assay on the automated thrombin generation instrument, the ST Genesia system. This instrument offers the capacity to continuously load patient samples for unitary testing which highly facilitates its use in clinical routine (Douxfils et al., 2019).

MATERIAL AND METHOD

General procedure of the ETP-based APCR assay

The ETP-based APCR assay has been extensively described in **section I.1** and is illustrated in the **Memento**.

Transferability of the ETP-based APCR assay on the ST Genesia system

The transferability, as described in **Figure I.3.1**, was performed as follows: (1)determination of the APC concentration to achieve 90% of ETP inhibition of the HPP, (2)assessment of the method precision, (3)definition of acceptance ranges for the reference plasma (STG-RefPlasma TS) and quality controls (STG-QualiTest Norm TS and STG-QualiTest High TS), (4)computation of the nAPCsr correction factor, (5)definition of the normal range and assessment of the sensitivity towards different oral contraceptive therapies and (6)comparison of the methods between the CAT and the ST Genesia system.

Determination of the APC concentration

The first step consisted of defining the amount of exogenous APC to be added in the TS reagent for the +APC condition. To this end, dose-response curves were performed using the in-house reference plasma, i.e., the HPP, and 2 different batches of TS (batch Nr 202983 and Nr 202984). To constitute the HPP, 20 healthy volunteers (10 men and 10 women not taking hormonal therapy) were enrolled and displayed the following characteristics: a mean age of 25 years (ranges, 18-56 years) and a mean body mass index (BMI) of 22 $kg.m^{-2}$ (ranges, 19-26 $kg.m^{-2}$). Five solutions of TS containing increasing concentrations of APC, i.e., 200 mU/mL; 400 mU/mL; 600 mU/mL; 700 mU/mL and 1034 mU/mL (final concentration in the reagent) were prepared to generate dose-response curves; all concentrations being derived from the APC labelled activity titre provided by the manufacturer. The concentration leading to 90% of ETP inhibition of the HPP was, then, interpolated for both batches of TS. Analyses were performed in triplicates on each batch of TS. It was then validated by analysing the HPP, 5 times within a same run, with both batches of TS in presence of the determined adequate APC concentration. The mean ETP inhibition percentage of the HPP obtained with each batch of TS had to stand between 87.5% and 92.5%.

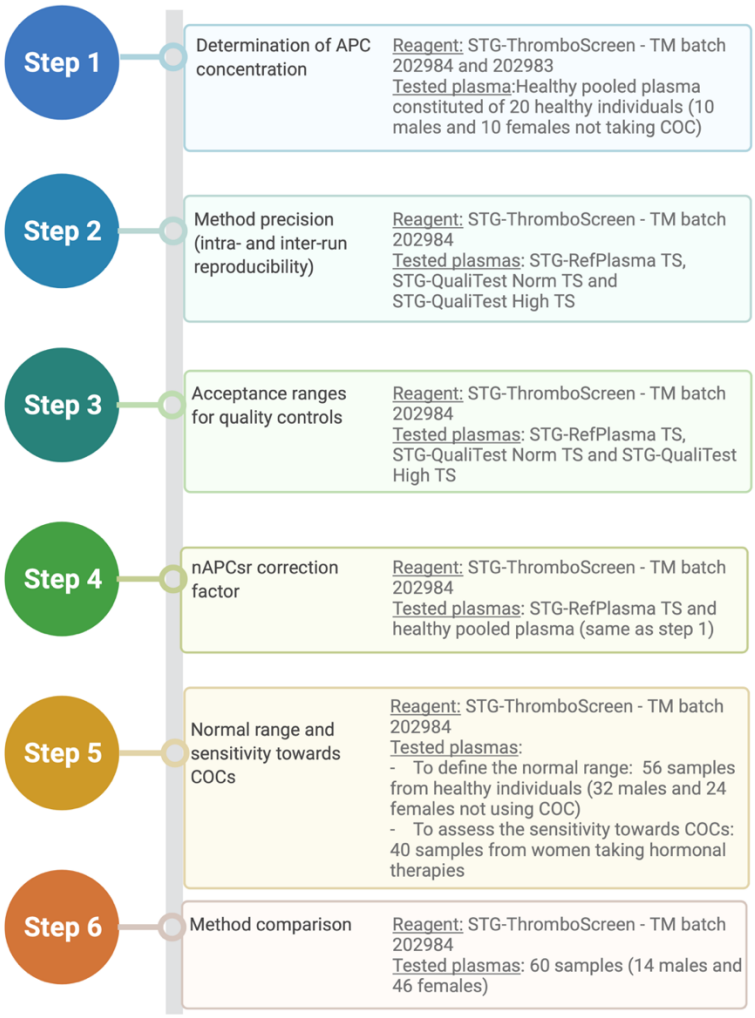


Figure I.3.1 | Flowchart of the transferability of the ETP based APCR assay on the ST Genesia system.

Assessment of the method precision

Intra- and inter-run reproducibility was assessed on 3 samples: the STG-RefPlasma TS, the STG-QualiTest Norm TS and the STG-QualiTest High TS. The third level of quality control (STG-QualiTest Low TS) included in the commercial kit was not analysed as its thrombin generation was completely inhibited in presence of APC.

The intra-run reproducibility was assessed by performing 5 individual measurements of the 3 above-mentioned plasmas, within the same run. For the inter-run reproducibility, those plasmas were analysed over 10 independent runs. Results

were expressed as ETP inhibition percentages and acceptance criteria were the same as for the method validation on the CAT system, i.e., standard deviation (SD) values lower than 10% (**Part I.1**). For this performance and subsequent steps, only one batch of STG-ThromboScreen was used (batch Nr 202984).

Definition of acceptance ranges of the commercial reference plasma and quality controls

Acceptance ranges have been defined as previously. For quality controls (i.e., STG-QualiTest Norm TS and STG-QualiTest High TS), ranges were computed as the mean ETP inhibition percentage (of 10 independent runs) ± 2*SD and 3*SD to follow Westgard rules. On the other hand, for the commercial reference plasma, the acceptance range was defined as the ETP inhibition percentage that corresponded to 87.5% and to 92.5% of ETP inhibition of the in-house HPP. This narrow acceptance range was defined to ensure a ratio close to 0.1 at the denominator, when computing the nAPCsr, to limit the nAPCsr score up to 10.

Determination of the nAPCsr correction factor

To compute nAPCsr values of plasma samples, the correction factor was determined based on the 10 runs of the commercial reference plasma performed during the inter-run reproducibility and on the 5 runs of the HPP performed during the APC concentration validation.

Definition of the normal range and assessment of the sensitivity towards different oral contraceptive therapies

To define the normal range, 56 samples from healthy individuals (32 men and 24 women not using hormonal therapy) were analysed. The mean age of participants was 23 years (ranges, 18-56 years) and the mean BMI was 23 $kg.m^{-2}$ (ranges, 17-32 $kg.m^{-2}$). Results were expressed in nAPCsr values. The lower and upper limit of the normal range corresponded to the 10-90th percentile of nAPCsr values.

To assess the sensitivity of the test in a subset of real-life samples, 40 healthy young women taking hormonal therapies were recruited and stratified in 3 subgroups, namely users of 2nd generation COCs (i.e. containing 20 or 30 µg of ethinylestradiol (EE) with 100 or 150 µg of levonorgestrel) [n=18; mean age, 21 years; mean BMI, 22 $kg.m^{-2}$], users of 3rd generation COCs (i.e. containing 20 or 30 µg of EE with 150 µg of desogestrel or 75 µg of gestodene) [n=13; mean age, 22 years; mean BMI, 22 $kg.m^{-2}$] and users of the so-called "other" COCs (i.e. containing 20, 30 or 35 µg of EE with 3 mg of drospirenone, 2 mg of cyproterone acetate or 2 mg of dienogest) [n=9; mean age, 21 years; mean BMI, 21 $kg.m^{-2}$]. The demographic characteristics (age and BMI) of the 3 groups were well matched. Comparisons between healthy individuals (men and women separated) and the 3 subgroups according to the use of hormonal

therapies were performed. Results were expressed as nAPCsr values and ETP values (for the latter, in absence and presence of APC).

Comparison between the CAT system and the ST Genesia system

Sixty samples from individuals (14 men and 46 women) were analysed with the validated assay on the CAT system and on the ST Genesia system. The mean age of participants was 21 years (ranges, 18-30 years) and the mean BMI was 22 $kg.m^{-2}$ (ranges, 17-31 $kg.m^{-2}$). To cover a wide range of nAPCsr values, of the 46 women, 13 were not using any contraception, 33 were on various hormonal contraceptives and finally, one participant was carrying a heterozygous FVL mutation and another one, a heterozygous prothrombin (PT) G20210A mutation. The comparison between both platforms was performed using nAPCsr results.

Blood samples collection and plasma preparation

All the volunteers included in this study were recruited at the University of Namur, Belgium. Biological samples were collected in accordance with the Declaration of Helsinki after approval by the Ethical Committee of the Centre Hospitalier Universitaire (CHU), Université Catholique de Louvain (UCL) Namur (Yvoir, Belgium) under the approval number B03920096633. Written informed consent was obtained from each donor. Exclusion criteria were history of thrombotic and/or haemorrhagic events, treatment by antiplatelets or anticoagulant medications or other drugs potentially affecting platelets or coagulation, pregnancy, use of hormonal therapy (only for the recruitment of healthy volunteers and for the constitution of the HPP), and carriers of FVL or PT G20210A mutations (except for the comparison between platforms). The absence/presence of FVL and PT G20210A mutations were identified by a CE-IVD-approved technique (LaCAR MDx Technologies S.A., Liège, Belgium). All samples were stored and managed by the Namur Biobank-eXchange (NAB-X), the registered biobank from the University of Namur.

Regarding sampling procedures, refer to the "*blood sample collection and plasma preparation"* in **Part I.2.**

Statistical analyses

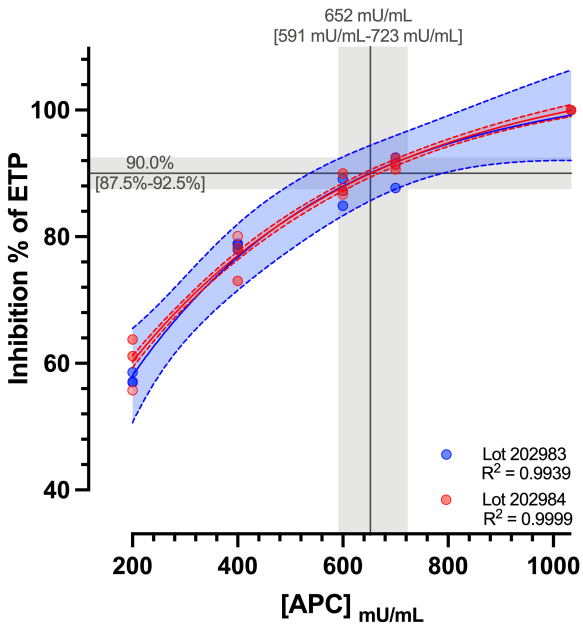
Statistical analyses were performed using GraphPad Prism version 9.0.0 for MacOs (GraphPad Software, San Diego, California, USA, www.graphpad.com). Descriptive statistics were used to analyse the data. The APC concentration was determined using non-linear regressions (one-phase association – least square fit) with no constrain on the Y-axis. As the SD is stable along the whole range of measurements, intra- and inter-run reproducibility was expressed in terms of SD instead of the coefficient of variation (CV). To compare subgroups with each other, ordinary one-way ANOVAs with Tukey's multiple comparison tests were performed. A Wilcoxon

matched-pairs signed rank test was performed to compare nAPCsr values on the ST Genesia versus the CAT system. A linear regression and a Spearman correlation were used to assess the correlation between the CAT system versus the ST Genesia system. A derived Bland-Altman analysis was performed by plotting nAPCsr differences (CAT – ST Genesia) against nAPCsr values obtained on the CAT system, considered as the gold standard. Within each subgroup, paired t-tests (or Wilcoxon matched-pairs signed rank tests) were performed to compare nAPCsr values between platforms. Finally, a two-way ANOVA with multiple comparison tests was performed to compare subgroups with each other.

RESULTS

Determination of APC concentration

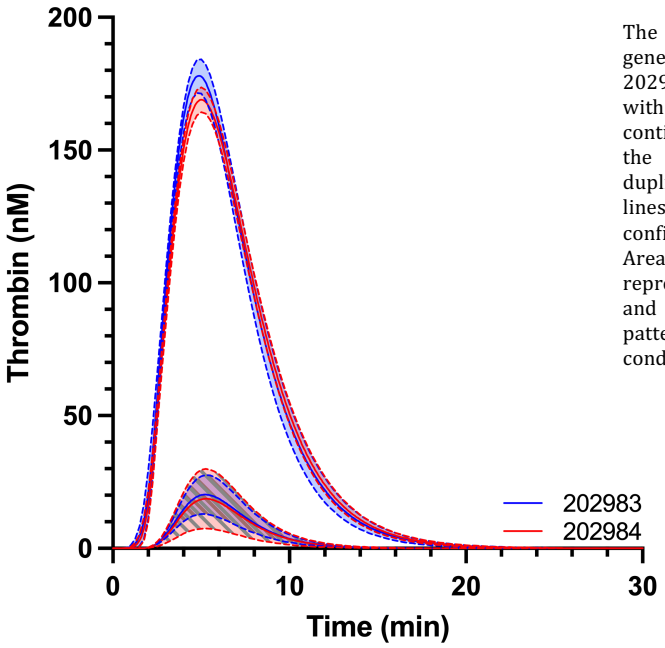
Dose-response curves of APC, performed on two different batches of TS, are shown in **Figure I.3.2**. Coefficients of determination R^2 of individual non-linear regressions were ≥0.98 with both batches of TS. The concentration of APC leading to 90% of ETP inhibition on both batches of TS was the same, i.e., 652 mU/mL. The intra-run reproducibility of the HPP tested using this APC concentration revealed a mean ETP inhibition percentage (±SD) of 91.3% (±4.8%) and 90.9% (±2.7%) on batch Nr 202984 and Nr 202983, respectively.



Dose-response curves performed with batch 202983 are represented in blue and those performed with batch 202984 are represented in red. The dark gray horizontal line and the light gray area on either side of the line represent the targeted inhibition percentage [lower-upper limit of acceptance], i.e., 90% [87.5%-92.5%]. The dark gray vertical line and the light gray area on either side of the line correspond to the APC concentration leading to 90% of ETP inhibition [upper-lower limit of acceptance], i.e., 652 mU/mL [591 mU/mL -723 mU/mL].

Figure I.3.2 | Mean inhibition percentages of the ETP of a healthy pooled plasma (HPP), at various concentrations of APC on two different batches of STG-ThromboScreen -TM (Nr 202983 and Nr 202984).

Thrombin generation curves of HPP, in absence and in presence of 652 mU/mL of APC, with both batches of TS, are shown in **Figure I.3.3**.



The blue curves were generated with TS batch 202983 and the red curves with batch 202984. The continuous lines represent the mean of the five duplicates and the dotted lines represent the 95% confidence interval (95%IC). Areas in absence of pattern represent the -APC condition and areas with the fill gray pattern represent the +APC condition.

Figure I.3.3 | Thrombin generation curves of a healthy pooled plasma (HPP), in absence and in presence of APC [652 mU/mL] with two different batches of STG-ThromboScreen -TM.

Method precision: intra- and inter-run reproducibility

Intra-run and inter-run reproducibility are shown in **Table I.3.1**. Standard deviation values for each tested plasma (i.e., STG-QualiTest Norm TS, STG-QualiTest High TS and STG-RefPlasma TS) were within acceptance criteria, i.e., SD ≤ 10%.

Table I.3.1 | Intra-run and inter-run reproducibility of STG-RefPlasma TS, STG-QualiTest Norm TS and STG-QualiTest High TS.

Intra-run reproducibility				
Tested plasma	**Replicate**	**ETP inhibition %**	**Mean ETP inhibition %**	**SD%**
STG-RefPlasma TS	1	74.2%	73.5%	1.0%
	2	74.0%		
	3	73.2%		
	4	74.2%		
	5	71.9%		
STG-QualiTest Norm TS	1	36.5%	38.5%	2.0%
	2	38.0%		
	3	37.2%		
	4	41.6%		
	5	39.2%		

Table I.3.1 (continued) | Intra-run and inter-run reproducibility of STG-RefPlasma TS, STG-QualiTest Norm TS and STG-QualiTest High TS

STG-QualiTest High TS	1	11.2%	**8.7%**	**1.7%**
	2	6.8%		
	3	7.8%		
	4	9.7%		
	5	8.3%		
Inter-run reproducibility				
Tested plasma	**Replicate**	**ETP inhibition %**	**Mean ETP inhibition %**	**SD%**
STG-RefPlasma TS	1	80.5%	74.5%	3.5%
	2	73.4%		
	3	74.8%		
	4	76.4%		
	5	68.7%		
	6	75.8%		
	7	75.1%		
	8	77.8%		
	9	72.7%		
	10	70.0%		
STG-QualiTest Norm TS	1	42.3%	37.7%	3.5%
	2	38.9%		
	3	38.7%		
	4	32.4%		
	5	33.3%		
	6	39.6%		
	7	40.5%		
	8	39.6%		
	9	38.7%		
	10	32.7%		
STG-QualiTest High TS	1	8.1%	10.1%	1.4%
	2	8.1%		
	3	10.4%		
	4	11.1%		
	5	10.7%		
	6	10.0%		
	7	9.1%		
	8	11.9%		
	9	9.6%		
	10	12.0%		

Acceptance ranges of the commercial reference plasma and quality controls

Acceptance ranges for the reference plasma and the two levels of quality control are shown in **Figure I.3.4.** The following Westgard rules were applied to monitor the run performances throughout the entire study: i) warning if one measurement exceeded 2*SD for one quality control (1_{2s}) and ii) rejection if one measurement exceeded

3*SD for one quality control (1_{3s}) or outside the acceptance range for the reference plasma. As depicted, no analysis was rejected throughout the course of the study. Nonetheless, on run 3, ETP inhibition percentages of STG-QualiTest Norm TS and STG-Qualitest High TS exceeded 2*SD but stood within the mean±3*SD.

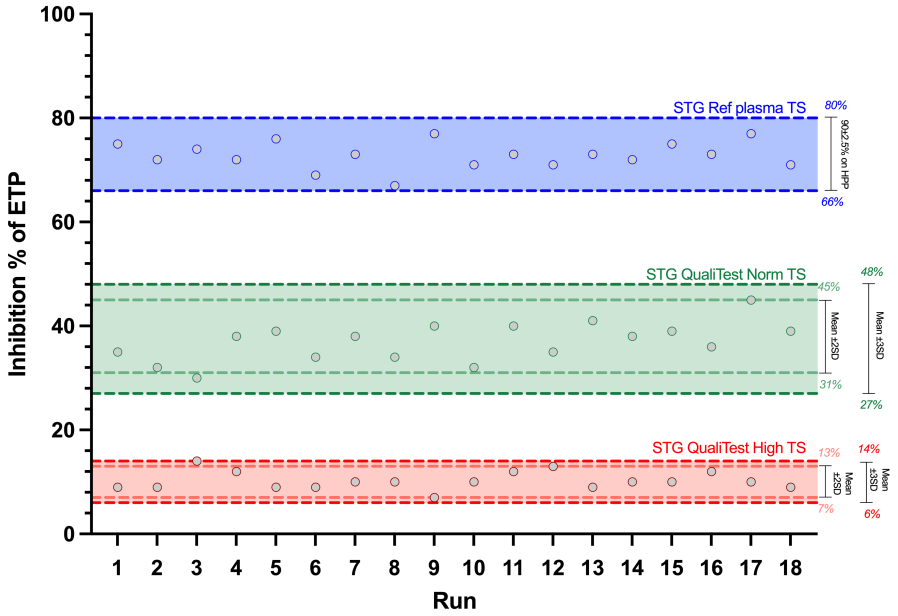


Figure I.3.4 | Quality controls (i.e., STG QualiTest Norm TS and QualiTest High TS) and reference plasma (i.e., STG-RefPlasma TS) follow-up.
The acceptance range for the STG-RefPlasma TS (blue dotted lines) stood from 66% to 80% of ETP inhibition. Acceptance ranges for STG QualiTest Norm TS (green dotted lines), corresponding to the mean ± 2*SD and the mean ± 3*SD, equalled [31%-45%] and [27%-48%] respectively. Acceptance ranges for STG QualiTest High TS (red dotted lines), corresponding to the mean ± 2*SD and the mean ± 3*SD, equalled [7%-13%] and [6%-14%] respectively.

Computation of the nAPCsr correction factor

The correction factor, which corresponded to the nAPCsr of the commercial reference plasma (issued from STG-ThromboScreen kit batch Nr 202984), using the HPP as the reference plasma, equalled 2.45. This correction factor was applied to compute the nAPCsr of all plasma samples, as described in **Equation 9**.

Equation 9

$$\text{Adjusted nAPCsr} = \frac{Sample\ ETP\ (+APC) \Big/ Sample\ ETP\ (-APC)}{STG-RefPlasma\ TS\ ETP\ (+APC) \Big/ STG-RefPlasma\ TS\ ETP\ (-APC)} * F \quad (2.45)$$

Normal ranges and sensitivity towards different oral contraceptive therapies

In the normal range study involving 56 healthy individuals, the mean nAPCsr was 1.03 [SD, 0.82; 95%CI of the mean, 0.82-1.25). The 10-90th percentile, chosen as the lower and upper limit for the normal range, equalled 0.00 and 2.20. When the gender was considered, men were significantly less resistant to APC than women with a mean nAPCsr of 0.69 and 1.49 respectively. Among the different COC-user groups, mean nAPCsr values (±SD) equalled 3.61 (±1.04) for 2nd generation COC users; 3.83 (±1.03) for 3rd generation COC users and 5.54 (±0.73) for the so-called "other" COC users. Tukey's multiple comparison tests showed significant differences between each subgroup except for women using 2nd and 3rd generation COCs (P>.05) (**Figure I.3.5**).

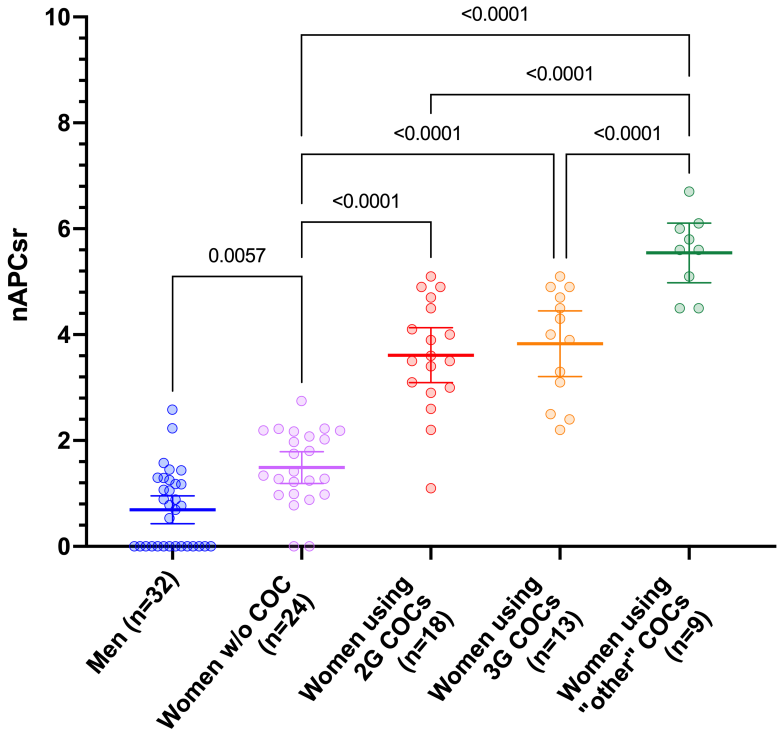
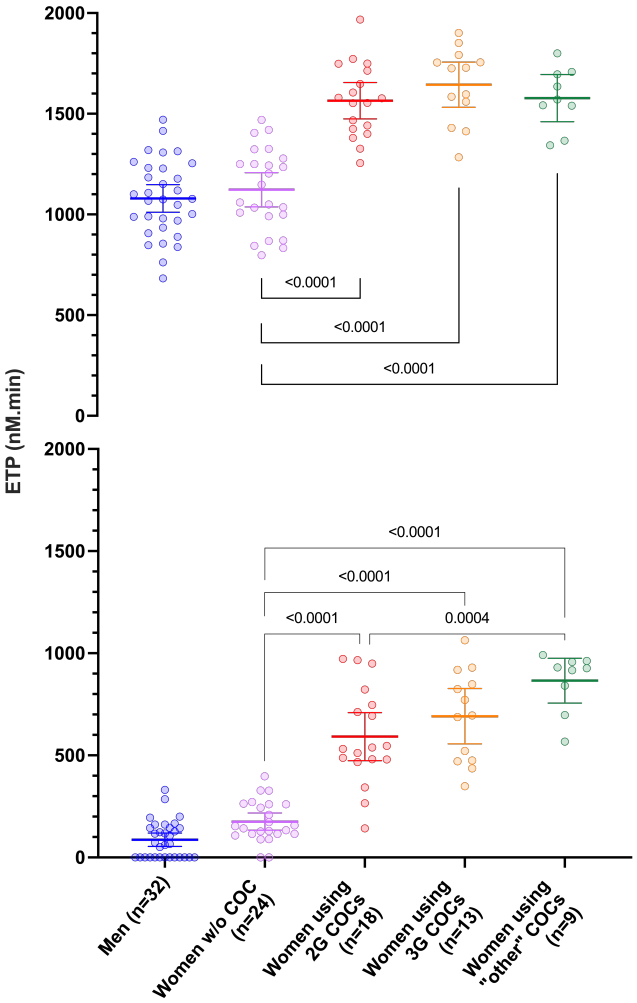


Figure I.3.5 | nAPCsr values of individuals from each subgroup, i.e., men (in blue), women not using hormonal contraception (in purple), women using 2nd generation COCs (in red), women using 3rd generation COCs (in orange) and women using the so-called "other" COCs (in green)
Individual's values and means ± SDs for each subgroup are represented. Differences between subgroups were assessed by an ANOVA with Tukey's multiple comparison tests. Threshold for significance was set at 0.05.

Regarding the ETP-parameter, values from individuals and means ±SDs in absence and presence of APC of each subgroup are shown in **Table I.3.2** and **Figure I.3.6**. In the absence of APC, no significant difference was observed between men and women not using COC, as well as among women using COCs (P>.05). Differences between healthy individuals (men and women not using COC) and women using COCs (regardless of the therapy) were significant. In presence of APC, the trends remained similar, excepted between women using 2nd generation COCs and women using "other" COCs, for which the difference was significant (P=.0004) (**Figure I.3.6**).



Individual's values and means ± SDs for each subgroup are represented. Differences between subgroups were assessed using an ANOVA with Tukey's multiple comparison tests. Threshold for significance was set at 0.05.

Figure I.3.6 | ETP values, in absence (upper graph) and in presence (lower graph) of exogenous APC of individuals from each subgroup, i.e., men (in blue), women not using hormonal contraception (in purple), women using 2nd generation COCs (in red), women using 3rd generation COCs (in orange) and women using the so-called "other" COCs (in green).

Table I.3.2 | Mean ETP values ±SD of the different subgroups in absence and in presence of APC in the ThromboScreen -TM reagent.

	ETP (-APC) ±SD (nM*min)	ETP (+APC) ±SD (nM*min)
Men (n=32)	1080 ±190	87 ±91
Women w/o COC (n=24)	1123 ±201	176 ±101
Women using 2G COCs (n=18)	1565 ±182	592 ±236
Women using 3G COCs (n=13)	1644 ±185	692 ±224
Women using "other" COCs (n=9)	1578 ±152	866 ±142

Comparison between the CAT and the ST Genesia system

To compare results obtained on the CAT system and the ST Genesia system, 60 plasma samples were analysed on both platforms. The Spearman correlation depicted in **Figure I.3.7** showed a significant effective pairing between both platforms. The correlation coefficient (Rs) was 0.9608 (95% CI, 0.9341 to 0.9769; P<.0001). Linear regression showed the following equation Y = 0.9529 x + 0.2013 (R^2 = 0.90). When nAPCsr differences (CAT system – ST Genesia system) were plotted against nAPCsr values from the CAT system (considered as the reference method as present study assesses its transferability), the mean difference between both platforms equalled -0.1 (95% CI, -1.3% to 1.1%) (**Figure I.3.8**).

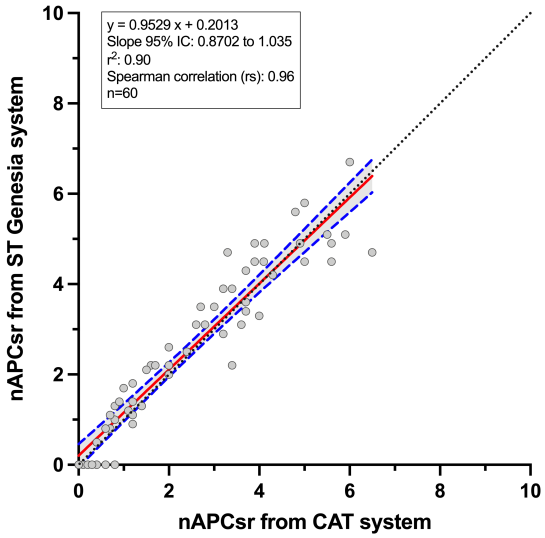


Figure I.3.7 | Correlation between nAPCsr values obtained on the ST Genesia system and on the validated platform, the CAT system.

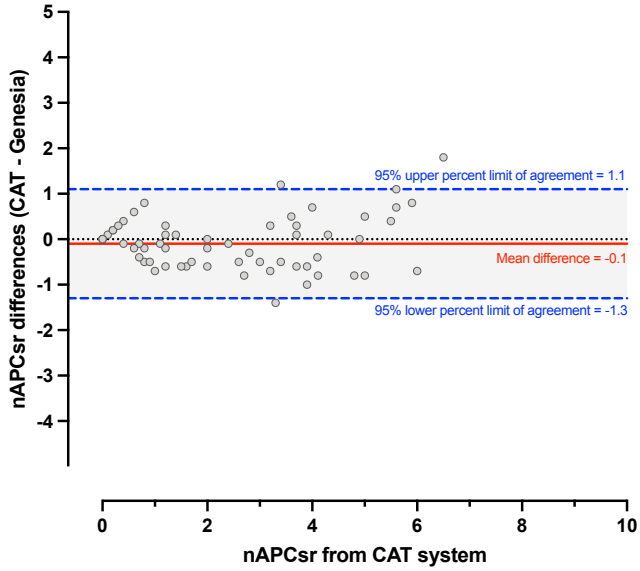


Figure I.3.8 | Derived Bland-Altman analysis between nAPCsr values obtained on the ST Genesia system and on the CAT system.
nAPCsr differences (CAT – ST Genesia) are expressed in absolute values. The mean difference (red continuous lines) ±95%CI [=1.96*SD) (blue dotted lines) equalled -0.1 ±1.2.

Comparisons between the CAT system and the ST Genesia system within subgroups (i.e., men, women not using COC, women using 2nd generation, 3rd generation or "other" COCs) are shown in **Figure I.3.9**.

As there was only one individual carrier of a PT G20210A mutation and another one carrier of a FVL mutation, no comparison with these groups was performed. Nevertheless, results were similar between the two systems, with nAPCsr values of 2.8 on the CAT versus 3.1 on the ST Genesia system for the PT G20210A mutation, and 4.3 on the CAT versus 4.2 on the ST Genesia system for FVL mutation.

Paired t-tests did not reveal any significant difference between the two systems within men, women not using COCs, women using 2nd and women using 3rd generation COCs (P>.05). As for women using "other COCs", size sample was too small. A Wilcoxon paired-t test was therefore performed, and no significant difference was observed between platforms (P>.05). On the other hand, the two-way ANOVA with multiple comparison tests showed significant differences between subgroups except for comparison between women using 2nd generation and 3rd generation COCs (P=.1412), and between women using 3rd generation and "other" COCs (P=.2337).

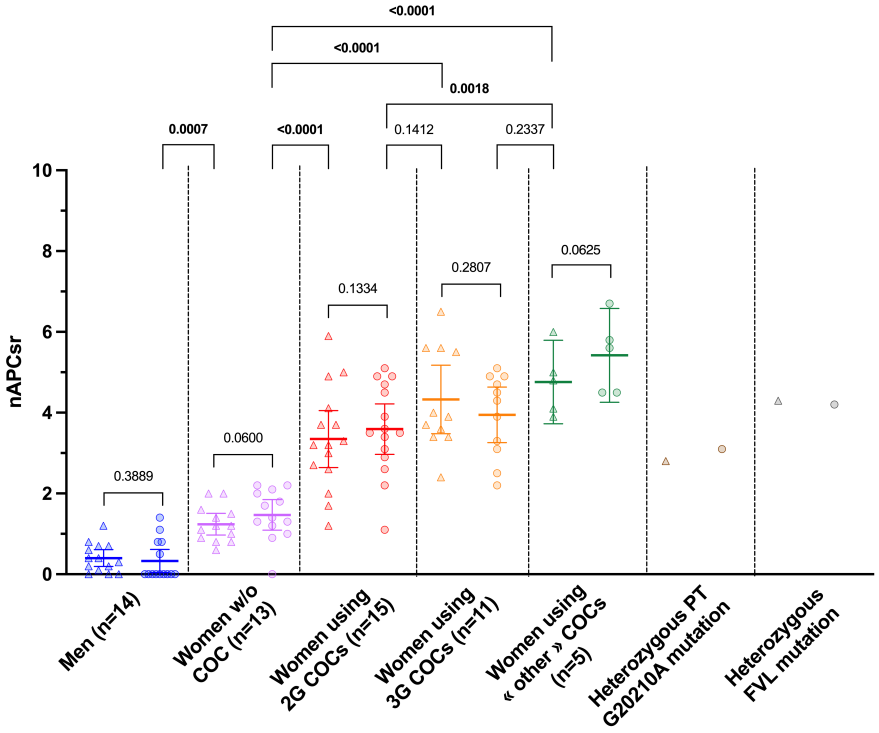


Figure I.3.9 | nAPCsr values of individuals from each subgroup, on both systems, i.e., the CAT system (triangle pattern) and the ST Genesia system (circle pattern)
Individual's values and means ± SDs for each subgroup are represented. Differences between systems within each subgroup were assessed using paired t-tests except for subgroup "women using other COCs, for which a Wilcoxon matched-pairs signed rank test was performed. On the other hand, differences between subgroups were assessed using a two-way ANOVA with multiple comparison tests. Threshold for significance was set at 0.05.

DISCUSSION

This is the first study evaluating the analytical performances of the ETP-based APCR assay on the ST Genesia system, an automated thrombin generation instrument for the routine setting (Douxfils et al., 2019). To date, the CAT system is probably one of the most used thrombin generation techniques in research laboratories but the use of microtiter plates, the manual placing of the reagent and the sample into the wells, have prevented its introduction into the routine practice (Tripodi, 2016). Nevertheless, as the ETP-based APCR could be a potential candidate as a biomarker of thrombogenicity in women on hormonal therapy or during pregnancy (Brenner, 2004; Cumming et al., 1995; Tchaikovski et al., 2011), it is crucial to facilitate its use in clinical routine and automation is the first stage for this.

The first step, which consisted of determining the concentration of APC to be added into the TS for the APC positive condition, was critical. Indeed, the source of the

exogenous APC differed from the one used with the validated methodology on the CAT system (**Part I.1**). Pre-tests therefore had to be performed to evaluate the inhibition capacities of the exogenous APC and to determine APC concentrations to perform dose/response curves. We decided to perform these dose/response curves with two different batches of STG-ThromboScreen -TM to assess the inter-batch variability of this reagent in presence of exogenous APC. Results showed that the chosen APC concentration of 652 mU/mL fitted for both batches (**Figure I.3.2**), as it led to 90.9% of ETP inhibition of the HPP with batch Nr 202983 and 91.3% with batch Nr 202984.

Regarding the method precision, the within- and between-run reproducibility showed maximal SD of 2.0% and 3.5% respectively, well below the maximal tolerable limit of 10%. In addition, SDs were similar on the entire range of measurements demonstrating a same precision of the assay, irrespective of the level of inhibition of the sample. Compared to the application on the CAT system, these results are highly satisfactory as maximal SD obtained with the validated methodology were 3.0% and 7.0% for the intra- and inter-run reproducibility respectively (**Part I.1**).

The daily management of the two quality control levels and the reference plasma provided acceptable results **(Figure I.3.4).** Their ETP inhibition levels were within the acceptance range among the 18 experiments performed throughout the study, except run 3, for which two QC levels stood between the mean ± 2*SD and ±3*SD.

The correction factor corresponding to the nAPCsr of the reference plasma equalled 2.45, indicating resistance towards the APC. As already mentioned, this may be related to the manufacturing process, e.g., the addition of excipients and the lyophilized form of the plasma. Indeed, it has already been reported that freeze-dried plasma has higher thrombin generation capacity than frozen plasma resulting from multiple preanalytical reasons and this was also observed regarding the sensitivity towards added protein C (Dargaud et al., 2010; Morimont, Bouvy, et al., 2020; Rodgers et al., 2014).

The analysis of 56 samples from healthy donors had two main objectives: first, to validate the correction factor of the commercial reference plasma, used for the computation of the nAPCsr. Second, to provide grounds for the definition of a normal range. The mean nAPCsr, i.e., 1.03, demonstrated that the correction factor for the commercial reference plasma was appropriate as a nAPCsr value close to 1 corresponds to an ETP inhibition % close to 90%, considered as the reference value for a healthy population. The nAPCsr normal range of 0.00 to 2.20 is also very close to the range previously defined on the CAT system corresponding to 0.00 to 2.08. This similar range indicates that the test, when performed on the ST Genesia system, gives comparable results to the CAT system in a population of healthy individuals.

Concerning the sensitivity, the assay was able to discriminate healthy subjects from women treated with COCs (i.e., 2nd generation, 3rd generation and the so-called "other" COCs). Of the healthy donors, we expected to observe a significant difference between men and women. Furthermore, women using COCs (i.e., 2nd, 3rd, or "other" groups) were significantly more resistant to APC, compared to both, men and women not using hormonal contraception. On the other hand, due to the low recruitment in the 3 COC subgroups, no significant difference was shown between 2nd and 3rd generation COCs (**Figure I.3.5**).

The final step of this transferability was the comparison between the two systems, i.e., the CAT system versus the ST Genesia system. Based on the linear regression and the derived Bland-Altman analysis, the ETP-based APCR assay, when performed on the automated platform, tended to give slightly higher nAPCsr values compared to the CAT system. Certainly, the ST Genesia system derived from the CAT principle but is not strictly equivalent (Giesen et al., 2021; Talon et al., 2020). The same fluorogenic substrate for thrombin is used on both systems but the calibration used to obtain thrombin concentration from the fluorescent signal is different: on the ST Genesia, thrombin generation is computed in comparison to a daily calibration curve obtained with a fixed amount of human purified thrombin (STG-ThrombiCal) in buffer solution in presence of the substrate Z-Gly-Gly-Arg-AMC and calcium (STG-FluoStart) (Giesen et al., 2021). The thrombin-mediated substrate cleavage leads to a fluorescent increase (AMC) which is measured every 15 seconds at 377 nm (excitation) - 450 nm (emission) wavelengths, which differs slightly from the CAT system sets to 390 nm (excitation)- 460 nm (emission) wavelengths (Giesen et al., 2021). Once the calibration passed, plasma samples are run individually, in duplicate. A known amount of AMC (STG-FluoSet) is added to each individual plasma sample for adjusting the calibration curve by correcting the plasma colour and rectifying the inner filter effect. The TG parameters are automatically calculated on both systems, but algorithms are instrument-specific which can therefore lead to differences in the results (Roullet et al., 2019; Talon et al., 2020).

LIMITATION AND PERSPECTIVES

This pilot study presented some limitations among which the absence of ready-to-use reagents. Indeed, the reagent TS + APC required the spiking of the APC into the TS reagent, which play a role in the inter-run variability. Moreover, it requires to redefine the APC concentration leading to 90% of ETP inhibition of HPP at each batch change of APC and/or STG-ThromboScreen kit. Indeed, the correction factor for the STG-RefPlasma TS will have to be computed again as well as acceptance ranges for quality controls. As a perspective, it is therefore of importance to consider the manufacturing of a kit like the STG ThromboScreen, thus containing a ready-to-use triggering reagent with APC, a reference plasma along with a correction factor for

the nAPCsr and the appropriate quality control levels. A second limitation was the low recruitment rate among women using hormonal therapies. Indeed, because of the small sample size, women were stratified by COC generation, whereas it is well documented that the risk of VTE differs within a COC generation depending on the dose of ethinylestradiol and the nature of the progestin (Morimont, Dogné, et al., 2020). One perspective is obviously to actively collaborate with hospitals to describe APCR of each COC according to its composition and no longer based on the so-called "generation" classification. It would also be relevant to consider the extent to which thresholds could be established in each population (e.g., women using ethinylestradiol 20µg with levonorgestrel 100µg) above which the thrombotic state of the patient would be considered as abnormal.

CONCLUSION

This study is the first reporting the analytical performances of the validated ETP-based APCR assay on the automated thrombin generation instrument, the ST Genesia system. The data revealed excellent intra-run and inter-run reproducibility, appropriate sensitivity towards hormonal therapies, and comparable results to those obtained with the validated methodology on the CAT system. This supports the concept that the nAPCsr, when performed into clinical routine, could become a promising regulatory and clinical tool to document on the thrombogenicity of female hormonal therapies and other coagulopathies interfering with the protein C system. In views of its screening potential, a complete validation on the ST Genesia system along with the manufacturing of ready-to-use triggering reagent with APC are essential and deserves further investigations.

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I.4 - LABORATORY TESTING FOR THE EVALUATION OF PHENOTYPIC APC RESISTANCE

Laure Morimont, Nathalie Donis, Céline Bouvy, François Mullier, Jean-Michel Dogné, Jonathan Douxfils

SUMMARY

Activated protein C (APC) resistance (APCR) is considered as a risk factor of venous thromboembolism (VTE). The most common genetic disorder conferring APCR is a factor V Leiden (FVL) mutation, but many other factors are also implicated, such as other *F5* mutations (e.g., FV Hong Kong and FV Cambridge), protein S deficiency, elevated factor VIII, exogenous hormone use, pregnancy and postpartum, depending on how APCR is defined. Considering the large population affected, the detection of this phenotype is crucial. Two types of tests are currently available: clotting time-based assays (with several versions) and thrombin generation-based assays with the endogenous thrombin potential (ETP)-based assay. The purpose of this review is therefore to discuss the performances of these tests and in which cases it would be appropriate to use one over the other. Initially, as APCR was thought to be solely related to the FVL mutation, the objective was to obtain a 100% specific assay. Clotting-time based assays were thus specifically designed to detect this inherited condition. Later on, an APCR condition without a FVL mutation was identified and highlighted as an independent risk factor of VTE. Therefore, the development of a less specific assay was needed. A global coagulation test was therefore proposed, known as the ETP-based APCR assay. In light of the above, these tests should not be used for the same purpose. Clotting time-based assays should only be recommended as a screening test for the detection of FV mutations prior to confirmation by genetic testing. On the other hand, the ETP-based APCR assay, in addition to being able to detect any type of APCR, could be proposed as a global screening test as it assesses the entire coagulation process.

INTRODUCTION

The complex formed by activated protein C (APC) and protein S plays a crucial role in the endogenous anticoagulant pathway. This system provides an important control over the blood coagulation cascade by inactivating activated factor V (FVa) and activated factor VIII (FVIIIa) (Dahlbäck, 2005; Dahlbäck et al., 2005). The activation of protein C takes place on the surface of endothelial cells and involves thrombin, thrombomodulin (TM) and the endothelial protein C receptor (EPCR) (**Figure I.4.1**).(Esmon, 2003) Thrombin promotes fibrin formation as well as platelet and endothelial cell activation but it also plays a role in the anticoagulant pathway by binding to TM to promote the activation of protein C. The EPCR then further amplifies the activation of protein C (Esmon, 2003). When APC is generated, it remains bound to the EPCR for a short time before associating with protein S on the surface of platelets or endothelium whereon it inhibits coagulation by degrading FVa and FVIIIa through proteolytic cleavage (Van de Wouwer et al., 2004). The proteolysis by APC occurs at amino acids position 306, 506 and 679 in FVa and 336, 562 and 740 in FVIIIa (Castoldi et al., 2004). Nevertheless, the cleavage of FVIIIa is not only carried out by the APC-protein S complex but also requires the inactivated form of FV as a cofactor (Dahlbäck et al., 2005). This pathway plays a key role in controlling coagulation and its disruption can easily unbalance homeostasis, causing a hypercoagulable state associated with an increased risk of thrombosis (Tans et al., 2003)

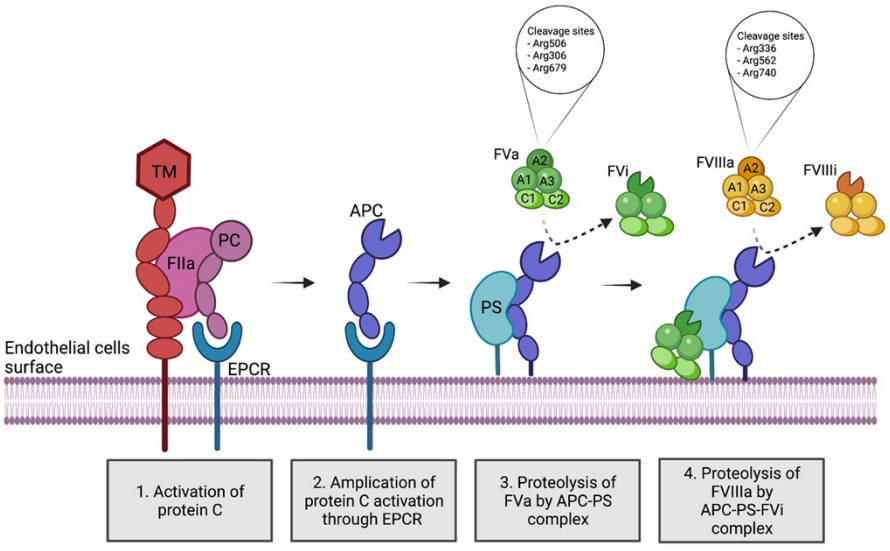

Figure I.4.1 | Protein C – protein S anticoagulant pathway

Functional defects in the protein C pathway, due to inherited or acquired conditions, define a plasma phenotype known as APC resistance (APCR), and is considered as a risk factor of venous thromboembolism (VTE) (Castoldi et al., 2004; de Visser et al., 1999; Rodeghiero et al., 1999; Tans et al., 2003). The most common genetic condition conferring APCR is the FV R506Q, better known as FV Leiden (FVL) mutation. A transition (guanine to adenine) at nucleotide 1691 in the gene coding for factor V results in the replacement of arginine (R) at position 506 by glutamine (Q). This substitution makes the 506 position less sensitive to proteolysis by the complex formed by APC with protein S. This slows the inactivation rate of FVa by 10-fold, resulting in an increased thrombin generation and a hypercoagulable state (Cramer et al., 2010; Dahlbäck, 1997; Khan et al., 2006; Kujovich, 2011). This genetic risk factor for VTE is found in 20% of patients with a first VTE event and in 50% of familial thrombosis (Kujovich, 2011). Heterozygosity for FVL occurs in 3-8% of the general US and European population, while homozygosity occurs at a frequency of about 0.02% (Hotoleanu, 2017; Khor et al., 2009). Compared to healthy individuals, the risk of first VTE event is 3-fold higher in heterozygous carriers whereas it may reach a relative risk (RR) of 30 to 80 in homozygous carriers (Germain et al., 2015; Hotoleanu, 2017).

Other less frequent mutations on the *F5* gene have also been identified and are FV Cambridge, FV Hong Kong, FV Bonn, FV Nara, FV Besançon, and FV Liverpool (Bernardi, 2016; Castoldi et al., 2021; Norstrom et al., 2002; Pezeshkpoor et al., 2016). FV Cambridge and FV Hong Kong affect the Arg306-cleavage site of FV. Arginine is replaced with threonine in FV Cambridge and with glycine in FV Hong Kong. Although FV Cambridge mutation is uncommon, the prevalence of FV Hong Kong is about 4% in Chinese population (Dahlbäck, 2004). Nevertheless, as the anticoagulant APC-cofactor activity of FV is essentially related to the 506-cleavage site rather than the 306-cleavage site, these mutations cause a moderate APCR and the increased risk of thrombosis associated with these 2 mutations remains unclear (Dahlbäck, 2004; Norstrom et al., 2002). Additional missense mutations such as the FV Besançon (Ala2986Asp), FV Liverpool (Ile358Thr), FV Nara (Trp1948Arg) or FV Bonn (Ala512Val) mutations had also been associated with an increased risk of thrombosis as they seemed to impact either FV levels or the anticoagulant activity of inactivated FV, therefore hampering the inactivation of FVIIIa (Castoldi et al., 2021; Nogami et al., 2014; Pezeshkpoor et al., 2016; Steen et al., 2004). Finally, the FV HR2 haplotype, characterized by multiple linked missense or silent mutations is associated with a slight decrease in circulating FV levels due to an impaired secretion of FV. The increased risk of thrombosis associated with this condition is ambiguous but combined with a heterozygous FVL mutation, the degree of APCR is similar to that observed in homozygous FVL carriers, hence characterized as pseudo-homozygous APCR (Aleksova et al., 2015; Bernardi et al., 1997; de Visser et al., 2000).

As protein S and FVIII are also part of this regulatory pathway, protein S deficiency or high FVIII levels could lead to an APCR phenotype. Hereditary protein S deficiency is a relatively rare disorder with a prevalence of 0.03-0.13% in the general population (although more frequent in Japan and China) but the associated RR of VTE was estimated between 5.0 and 11.5 compared to wild-type individuals (Hotoleanu, 2017; ten Kate et al., 2008). Regarding high FVIII levels (>150 IU/dl), a risk ratio of VTE of 4.8 was determined compared to normal individuals (FVIII levels<100 IU/dl) (Jenkins et al., 2012; Yap et al., 2015).

In addition, the hormonal status of women is also a condition which confers resistance to APC. Pregnancy and postpartum periods, as well as administration of exogenous hormones, such as combined hormonal contraceptives (CHCs) or menopausal replacement therapies (MRTs) during menopause, expose women to hormonal changes, and are also associated with an increased risk of VTE. Indeed, compared to non-pregnant nonusers, a 5-fold increased risk of VTE is reported during pregnancy, and depending on the estroprogestative association, the RR varies between 1.3 and 5.6 in women using CHCs or MRTs (de Bastos et al., 2014; Dinger et al., 2016; Heit et al., 2005; Jick et al., 1995; Lidegaard et al., 2009; Rovinski et al., 2018; Spitzer et al., 1996; van Hylckama Vlieg et al., 2009).

Pregnancy and the use of CHCs or MRTs cause changes in plasma levels of almost all proteins involved in coagulation and fibrinolysis (Douxfils, Morimont, & Bouvy, 2020). These changes might be considered as relatively modest when measured separately but they could have a supra-additive effect leading to a procoagulable state responsible for this increased risk of VTE (Morimont, Haguet, et al., 2021). Overall, rises in coagulation factors II, V, VII, VIII, IX, X, XI, XII and von Willebrand factor (VWF), as well as fibrinogen levels are observed. On the other hand, antithrombin, free protein S and tissue factor pathway inhibitor (TFPI) levels, 3 proteins contributing to the anticoagulant system, are decreased (Bonnar, 1987; Meade et al., 1977; Stadel, 1981; Wessler et al., 1976). As for fibrinolysis, there is an increase in plasminogen levels but a decrease in tissue plasminogen activator antigens and plasminogen activator inhibitor-1 levels (Douxfils, Morimont, & Bouvy, 2020). These hormonal changes, both during pregnancy and following the use of hormonal therapy, are also associated with APCR (Brenner, 2004; Morimont, Haguet, et al., 2021). This phenomenon has been first described in 1995 by *Henkens et al.*(Henkens et al., 1995) and *Olivieri et al.*(Olivieri et al., 1995) and is now largely documented. Afterwards, APCR became an important biomarker to evaluate the increased risk of VTE associated with CHCs, leading, in 2005, to the Committee for Medicinal Products for Human Use (CHMP) of the European Medicines Agency (EMA) stating that APCR should be investigated during the development of new steroid contraceptives in women (European Medicines Agency, 2005).

Given the countless number of people with APCR whether acquired or inherited, its detection is of great interest. Over the years, numerous assays have been developed and some of them might have the potential to be used as a screening tool with the aim to identify a hypercoagulable state, yet without determining the underlying cause. The purpose of this review is therefore to discuss the performances of these tests and in which cases it would be appropriate to use one over the other.

LABORATORY TESTING FOR APC RESISTANCE

Clotting time-based assay

The original assay for screening APCR was described by *Dahlbäck et al.* in 1993 and consisted of an activated partial thromboplastin time (aPTT)-based method to determine the sensitivity of a patient's plasma after the addition of exogenous APC (Dahlbäck et al., 1993). The aPTT assay is based on the principle that in citrated plasma, the addition of phospholipids (PLs), activator of FXII (e.g., micronized silica or ellagic acid) and calcium chloride triggers the formation of a stable clot. The time between activation and clot formation is recorded in seconds and represents the aPTT (Roshal et al., 2019).

When exogenous APC is added, the aPTT is prolonged, but in plasma from patients with APCR, this prolongation is less pronounced (Dahlbäck et al., 1993). Indeed, in case of a FVL mutation, the anticoagulant APC-protein S pathway is less effective, resulting in a shorter clotting time compared to a normal plasma in the presence of exogenous APC. Results are usually expressed as a ratio between the aPTT (+APC) and the aPTT (-APC). Values >2.0 are expected in normal populations while a FVL mutation typically gives a ratio <2.0. Nevertheless, each laboratory must verify its own cut-off since it may differ according to the kit used, as recently reported in several external quality control surveys (Dean et al., 2020; "External quality Control for Assays and Tests With a focus on Thrombosis and Haemostasis. Thrombophilia - II: APC Resistance (without FV deficient plasma) - Survey 2021-M1 (A). Version 2.0.0. 14 June 2021," ; Kadauke et al., 2014). Results may also be reported as normalized ratio, which is the ratio of the patient's APC ratio divided by the ratio of a normal pooled plasma (NPP) run on the same day; however, normalization against NPP does not improve the diagnostic performance, and unknown FV status in the donor population affects the accuracy (Tripodi et al., 1998). These external quality control surveys reported important discrepancies between the different kits on the market but also within a particular kit, revealing that improvements have to be done for the proper screening of FVL-induced APCR (Dahlbäck et al., 1993; Dean et al., 2020; "External quality Control for Assays and Tests With a focus on Thrombosis and Haemostasis. Thrombophilia - II: APC Resistance (without FV deficient plasma) - Survey 2021-M1 (A). Version 2.0.0. 14 June 2021,"). In addition, other FV mutations,

e.g., FV Hong Kong and Cambridge, may lead to intermediate APCR ratios compared to wild-type FV and FVL (Norstrom et al., 2002).

The very first aPTT-based APCR assay was subject to multiple variables, hampering the interpretation of the result. Anticoagulant drugs (e.g., vitamin K antagonists, heparins, direct FXa inhibitors and direct thrombin inhibitors) and factor deficiencies tended to falsely increase the APC ratio while protein S deficiency, lupus anticoagulant, pregnancy and hormonal therapy (e.g., MRTs, CHCs) tended to decrease it, leading to acquired APCR (Moore et al., 2019). To avoid these interferences and focus on the detection of FVL, modifications were implemented such as the dilution of the patient plasma with FV deficient plasma to become insensitive to abnormal levels of coagulation factors other than FV and the addition of a heparin neutralizer (e.g., polybrene). The normalization against NPP was also suggested to reduce intra- and inter-assay variability (Kadauke et al., 2014). Other alternative methods trigger the coagulation cascade directly through the activation of FX, by using either snake venom from *Crotalus viridis helleri* or from Russel Viper Venom (RVV-X, a snake venom extracted from *Daboia russelii*) or through the activation of FV by using RVV-V (also from *Daboia russelii*) plus Noscarin, a FV-dependent prothrombin activator extracted from *Notechis scutatus scutatus* (Moore, 2022). This latter assay is sometimes referred to as a prothrombin-based APCR assay (Gessoni et al., 2007; Schoni et al., 2007; Wilmer et al., 2004), and it does not require the presence of calcium ions and PLs therefore allowing to eliminate the influence of lupus anticoagulant (Wilmer et al., 2004). Beside these snake venom-based assays, the FXa-based assay is a test for which the patient's plasma is diluted in a proprietary reagent containing FII, fibrinogen, protein S and APC. Purified FXa, PLs and calcium are then added to initiate the coagulation (Jilma-Stohlawetz et al., 2021; Kadauke et al., 2014; Moore et al., 2019). In parallel, a calibration curve derived from dilutions of heterozygous FVL plasma pool converts clotting times to percentages. The prolongation of the clotting time is an inverse relationship to the FVL concentration present in the tested specimen. Normal FV presence does not affect this test.

The expected value in a FVL heterozygote stands between 25% and 75% and for a homozygous, it is above 75% (Jilma-Stohlawetz et al., 2021; Kadauke et al., 2014; Moore et al., 2019).

The activation pathways and clotting mixtures of these clotting-time based assays are shown in **Figure I.4.2** and the commercially available kits are listed in **Table I.4.1**.

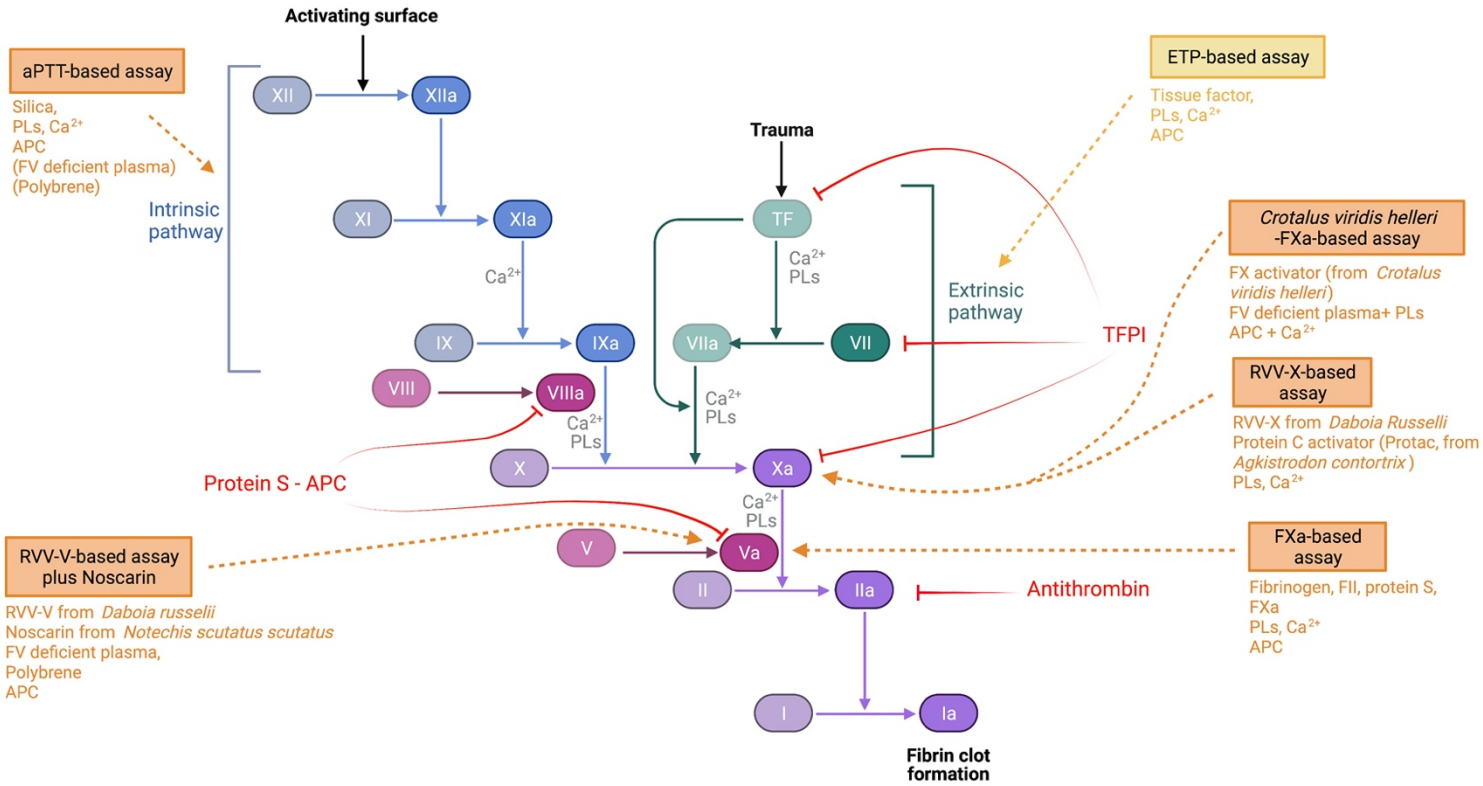

Figure I.4.2 | Simplified overview of coagulation cascade and APCR assay

Table I.4.1 | Commercially available clotting time-based assays for APCR evaluation

Assay name	Commercial kit	Laboratory
aPTT-based assay	HemosIL™ Factor V Leiden (APC™ Resistance V)	Werfen
	Chromogenix Coatest™ APC™ Resistance	Werfen
	Chromogenix Coatest™ APC™ Resistance – V	Werfen
RVV-X-based assay	ProC® Ac R Assay	Siemens
***Crotalus viridis helleri*-FXa based assay**	STA®-Staclot® APC R	Stago
RVV-V plus Noscarin	Pefakit® -APC-R Factor Leiden	Pentafarm
	Acticlot® Protein C resistance	Sekisui
FX-based assay	HemoClot™ Quanti-V Leiden	HYPHEN BioMed

These modified assays have been developed to detect the phenotype associated with a FVL mutation specifically, although other FV mutations may lead to APCR as well, and which may be variably identified in APCR assays, depending on assay sensitivities and the assay cut-offs used (Norstrom et al., 2002). Compared to genetic analysis using polymerase chain reaction technology, these functional tests are much less expensive and present a shorter turnaround time. Furthermore, as these clotting-time based assays show a sensitivity and a specificity of almost 100% for the detection of FVL mutation, it could make sense to abstain from genetic testing (Wilmer et al., 2004). However, their high specificity may be considered as a limitation as they may not be able to detect any resistance to APC, induced by either intrinsic or extrinsic factors (e.g., elevated FVIII levels, protein S deficiency, pregnancy and postpartum, or even the use of CHC and MRT), or other *F5* mutations (Castoldi et al., 2010; de Visser et al., 2000; de Visser et al., 2005; Graf et al., 2003)

For this reason, *Nicolaes et al.* developed in 1997 a new method to assess APCR resistance based on a global coagulation test instead of clotting time-based assay, known as the endogenous thrombin potential (ETP)-based APCR assay (Nicolaes et al., 1997).

Thrombin generation-based assay

The ETP-based APCR assay was developed to assess acquired APCR in pregnant women, women taking oral CHCs or MRTs, and in subjects with protein S deficiency, or carrying either a FVL or a prothrombin (PT) G20210A mutation (Curvers, Thomassen, et al., 2002).

This test is a variant of the thrombin generation assay, a global coagulation test enabling a continuous overview of clotting compared to the standard coagulation assays like aPTT, which retrieves only a clotting time result and represent ~5% of thrombin generation. The ETP-based APCR assay is based on the measurement of thrombin generation in presence and in absence of a defined amount of exogenous APC (Douxfils, Morimont, Delvigne, et al., 2020). The activation of coagulation occurs via the extrinsic pathway, following the addition of PLs, tissue factor (TF) and calcium (**Figure I.4.2**). In the absence of APC, the resulting thrombin generation curve reflects all the pro- and anticoagulant reactions that regulate both thrombin formation and inhibition. In the presence of APC, thrombin generation is significantly decreased in a normal plasma sample (i.e., ~90%). The endpoint of the test, which is the total amount of thrombin that has been generated over time, is quantitated by the ETP which corresponds to the area under the thrombin generation curve (**Figure I.4.3**).

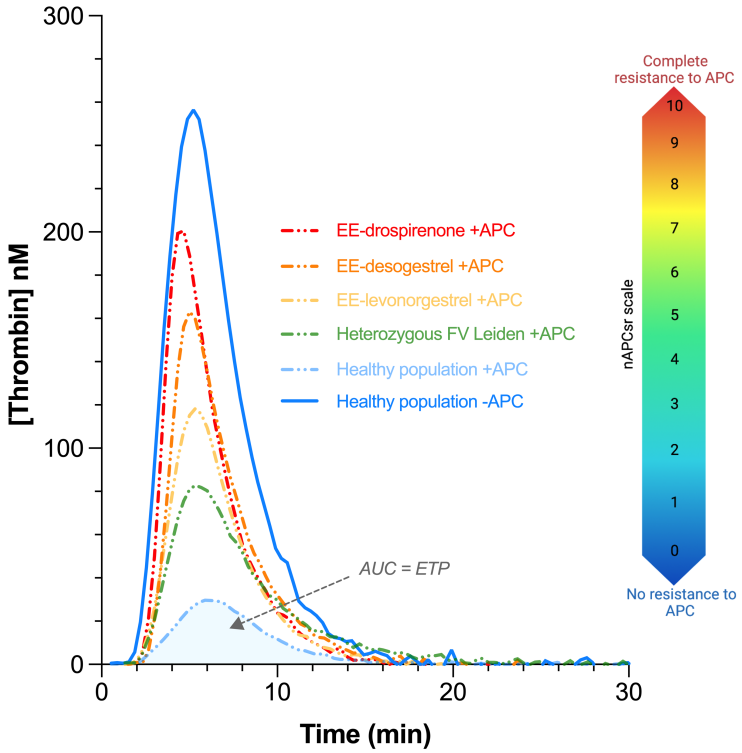

Figure I.4.3 | Thrombin generation curves in absence (continuous lines) and in presence of APC (dotted lines) of healthy donors (blue), of a woman carrier of a heterozygous FVL mutation (pink) and of women using COC containing either EE with levonorgestrel (orange), with desogestrel (yellow) or with drospirenone (green).

The amount of APC introduced in the test, to obtain a good sensitivity and to limit the inherent variability of the assay, targets a decrease of 90% of the ETP of a healthy

pooled plasma, i.e., a pool of plasma composed of men and women of childbearing age not using a hormonal contraceptive therapy. In other words, the ETP retrieved in presence of APC, represents only 10% of the baseline ETP (in the absence of APC) (Douxfils, Morimont, Delvigne, et al., 2020).

Results are expressed as a ratio, the normalized APC sensitivity ratio (nAPCsr), computed as the ratio of the ETP measured in presence and absence of APC in the tested plasma divided by the same ratio obtained from the reference plasma (e.g., a healthy pooled plasma) (**Equation 2**). As the denominator value is close to 0.1, this allows obtaining a scale ranging from 0 to 10. Importantly, conversely to the aPTT-based assay, the higher the nAPCsr, the more resistant the patient is to APC.

The test was initially performed on a thrombin generation system based on the cleavage of a chromogenic substrate specific for thrombin (Nicolaes et al., 1997). Because of technical and methodological difficulties limiting this technique (Tchaikovski et al., 2007) and because of the technological advance brought by the Calibrated Automated Thrombogram (CAT) developed by *Hemker et al*. in the early 2000s (Hemker et al., 2003), the ETP-based APCR assay was adjusted on a fluorometric technique (Brugge et al., 2006; Tchaikovski et al., 2007). However, the inherent limitations of the CAT system, such as the lack of standardization of the reagents, the absence of quality controls and reference plasma and the batch-to-batch variation, were also applicable for this assay (Dargaud et al., 2010; Dargaud et al., 2007; Loeffen et al., 2012; Perrin et al., 2015). Indeed, besides the differences imposed by a chromogenic or a fluorogenic assay, with the use of defibrinated plasma or platelet-poor plasma (PPP), respectively, differences in the source and concentration of TF, APC and PL vesicles, led to different sensitivities of the assays toward the APCR (Morimont, Bouvy, et al., 2020; Morimont, Haguet, et al., 2021). This hampered study-to-study comparisons which *in fine* impeded the proper evaluation of APCR induced by hormonal therapies or the evaluation of prothrombotic states. For this reason, this test has long been put aside.

In 2019, we proposed a standardized methodology that met all the standard requirements imposed on clinical biology laboratory tests in terms of analytical performances (i.e., Food and Drug Administration (FDA) “Guidance for Industry: Bioanalytical Method Validation”(Food and Drug Administration, 2018) and International Council for Harmonization (ICH) Q2 (R1) “Validation of Analytical Procedures: Text and Methodology”(European Medicines Agency, 1995)). The ETP-based APCR was validated on the CAT device using commercially available reagents to ensure batch-to-bath traceability, recovery and reproducibility of the method over time (Douxfils, Morimont, Delvigne, et al., 2020). This enabled the reduction of inter-laboratory variability and allowed lab-to-lab and study-to-study comparison and evaluation (Morimont, Didembourg, et al., 2021). Ultimately, this validation

provides pharmaceutical industries, regulatory bodies and physicians with a reproducible sensitive and validated assay that could be proposed as a gold standard for the assessment of all types of APCR.

This test, being performed in standardized conditions, quantifies the degree of APCR through the nAPCsr scale ranging from 0 to 10. As shown in **Figure I.4.3** , APCR, characterized by a higher thrombin generation curve in presence of APC, is observed in carriers of a FVL mutation as well as in women using CHCs. Besides, significant differences can be observed depending on oestrogen-progestin associations (e.g., levonorgestrel-containing products versus desogestrel- or drospirenone- containing products). In addition, the presence of both FVL mutation and CHC leads to a supra-additive effect which is reflected by higher nAPCsr values compared to CHC use or FVL mutation alone (**Figure I.4.4**).

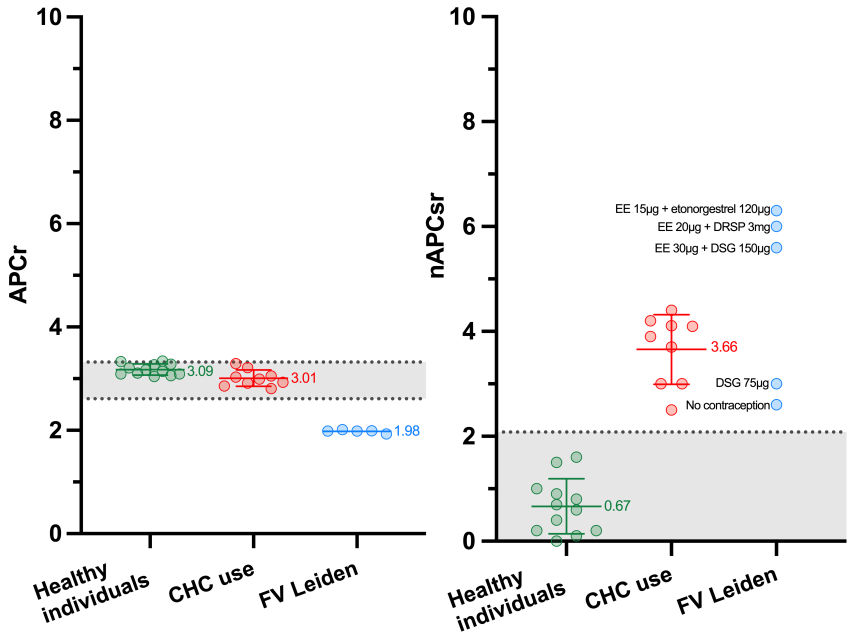

Figure I.4.4 | Assessment of APCR of healthy individuals (n=12), carrier of heterozygous FVL mutation(n=5) and women using COC (n=9), using (A) the aPTT-based (with predilution) or (B) the ETP-based APCR assays. Gray areas on both graphs represent normal ranges.

However, this test has some limitations. As well as the aPTT-based APCR assay, the ETP-based assay is sensitive to anticoagulants. The addition of polybrene in the triggering reagent allows neutralizing heparin in plasma samples, which allows restoring thrombin generation in presence of concentrations of unfractionated heparin and low molecular weight heparin (e.g., enoxaparin) up to 1.0 and 1.2 IU/mL

respectively (Hardy et al., 2021). Nonetheless, this has not yet been investigated in the presence of exogenous APC and deserves further investigations. Regarding the use of direct oral anticoagulants (DOACs), there are three commercially available DOAC removing agents: DOAC Stop™, DOAC Remove™ and DP-Filter™. However, these charcoal-based agents showed a slight procoagulant effect on thrombin generation at the medium TF concentration used in the ETP-based APCR assay. Indeed, in the absence of anticoagulants, they induced a higher peak, a higher mean velocity rate index and a lower time-to-peak compared to a non-treated plasma and this impact had been associated with a decrease in TFPI levels of plasma samples (Kopatz et al., 2018; Monteyne et al., 2020). As a result, diminished TFPI levels could impact the ETP-based APCR assay as this physiological anticoagulant protein plays an important role (de Visser et al., 2005). Indeed, TFPI inhibits FXa and subsequently TF and FVIIa by forming an inactive FXa-TFPI-TF-FVIIa quaternary complex. If TFPI levels decrease, FXa levels increase, therefore protecting FVa from inactivation by APC and reducing sensitivity for APC (de Visser et al., 2005).

According to the communication from the Scientific and Standardization Committee (SSC) of the International Society on Thrombosis and Haemostasis (ISTH), a current barrier to its use, which could be eliminated in the future, is the unavailability of this technique in clinical routine (Moore et al., 2019). Indeed, the validated methodology has recently been transferred on an automated thrombin generation platform, the ST Genesia system. Data revealed good reproducibility (standard deviations of 2.0% and 3.5% for within- and between-run reproducibility respectively), appropriate sensitivity towards hormonal therapies (significant differences between healthy individuals and women using various CHCs), and comparable results to those obtained with the validated methodology on the CAT system (Spearman correlation coefficient [95% confidence interval] of 0.9608 [95% CI, 0.9341 to 0.9769], based on 60 plasma samples) (Morimont, Leclercq, et al., 2022).

IN PRACTICE WHEN SHOULD THESE TESTS BE USED?

APCR testing is not indicated in unselected patients presenting with VTE and even less for the prevention of thrombotic events. Indeed, the evaluation of APCR is only recommended for situations in which the test result may give an indication of the recurrence risk or influence the anticoagulant treatment. Typically, it concerns patients suffering from VTE before the age of 40 and patients who are from apparent thrombosis-prone families (>2 other symptomatic family members) (Baglin et al., 2010).

As the FVL mutation is more common than acquired APCR and other *F5* mutations, the current recommendations call in first place, the use of clotting-time based assays with plasma predilution (Moore et al., 2019). However, APCR without FVL mutation

represents an independent risk factor of VTE (de Visser et al., 1999; Rodeghiero et al., 1999), so there is a value in having a less specific assay, able to detect any APCR phenotype. The ETP-based APCR assay is capable of doing so, which makes it less specific and therefore not able to identify the underlying cause of APCR.

Thus, both test types certainly assess APCR, but they should not be used for the same purpose. Moreover, inconsistent results, as shown in **Figure I.4.4**, are typically observed between these two assays. Clotting time-based assays should only be recommended as a substitute of genetic testing for FVL mutation and not for the evaluation of APCR during, for example, the development of steroid contraceptives in women, although it is mentioned in the EMA guidelines (EMEA/CPMP/EWP/519/98 Rev.1) (European Medicines Agency, 2005). In contrast, the ETP-based APCR assay has demonstrated, for over 20 years, its ability to detect any APCR phenotype, and especially, those associated with female hormonal changes, although it was not validated nor standardized until recently (Douxfils, Morimont, Delvigne, et al., 2020). This test not only detects the presence of acquired APCR but it also correlates with the relative risk of VTE associated with the different CHCs formulations available on the market (Morimont, Dogné, et al., 2020). Despite the fact that CHCs are used by over 150 million women worldwide (United Nations, 2019) no clear risk minimization strategy to manage the risk of VTE has been implemented. Overall, the absolute risk of VTE remains low (i.e., 5 to 12 for 10,000 women a year). However, given the large number of CHC users, 22,000 thrombosis related to CHC use would occur each year in Europe (McDaid et al., 2017). The worldwide use of MRT is certainly lower, but the annual incidence is higher with 3 to 4 cases of VTE per 1,000 women (Curb et al., 2006; Cushman et al., 2004; Rovinski et al., 2018).

As the field of personalized medicines is currently expanding, a biomarker capable of reflecting the “coagulability status” of individuals would be of great interest. Indeed, VTE is a multifactorial disease whose occurrence depends on the interaction between gene defects and environmental factors (Cosmi et al., 2003). As a result, exposure to high-risk situations such as surgery, trauma, immobilization, pregnancy, or hormonal therapy may trigger a thrombotic event in individuals either in the absence or presence of genetic mutations. This suggests that the evaluation of thrombophilia should be based on phenotypic expression rather than only focusing on genotypic expression.

The ETP-based APCR assay, reflecting all the pro- and anticoagulant reactions might be a potential candidate. Indeed, the typical information obtained by thrombin generation investigation (i.e., without exogenous APC added) is available, providing much more information than the APCR itself. As it enables a more global assessment of coagulation process, this assay is also sensitive towards other factors of

thrombogenicity like the PT G20210A mutation, antithrombin and protein S deficiencies or high FVIII levels (Duchemin et al., 2008; Lavigne-Lissalde et al., 2010; Marco et al., 2012; Szlam et al., 2011).

A method able to assess the entire coagulation process is valuable, as this can better reflect bleeding and thrombotic risks as compared to clotting time-based assays. Indeed, the endpoint of clotting-time based assays occurs after the formation of only 5% of total thrombin, which means that recorded clotting times only correspond to the initiation phase of the coagulation process (Duarte et al., 2017).

CONCLUSION

A global screening test could be the key to detect prothrombotic phenotypes associated with an increased risk of VTE. The ETP-based APCR assay, by considering the entire coagulation process and assessing thrombin generation in two different conditions (with and without exogenous APC), allows the identification of a large panel of prothrombotic states. Currently, evaluation of a complete thrombophilia panel requires multiple coagulation tests, which can make the interpretation of the results expensive and challenging. Indeed, changes in coagulation factors levels may not exceed their respective normal ranges, when assessed individually. On the other hand, the increased thrombogenicity, resulting from the additive effect of these changes could be captured by the ETP-based APCR assay. This represents an interesting approach that needs further clinical validation to assess and score a hypercoagulable state that would help the clinician in decision-making.

PART II: CORRELATION BETWEEN nAPCSR AND THE RELATIVE RISK OF VENOUS THROMBOEMBOLISM IN WOMEN USING COMBINED ORAL CONTRACEPTIVES

Thrombosis Research. 2020; 193

II.1 - RESPONSE TO THE ARTICLE OF ABOU-ISMAIL, ET AL. ENTITLED *"OESTROGEN AND THROMBOSIS: A BENCH TO BEDSIDE REVIEW"*

Laure Morimont, Jean-Michel Dogné, Jonathan Douxfils

To the Editors,

We read with great interest the excellent review of Abou-Ismail *et al.* on oestrogen and thrombosis (Abou-Ismail et al., 2020) and we would like to take the opportunity to further elaborate on this topic considering the latest data from in vitro diagnostic and drug candidates in development.

The management of venous thromboembolic events (VTE) related to combined oral contraceptives (COCs) use remains controversial. More than 100 million women worldwide are at risk and to date, no clear risk minimization strategies have been implemented. Overall, the absolute risk of VTE remains low (i.e., 5 to 12 for 10,000 women a year). However, in view of the incidence of thrombosis among the large number of COC users, it is estimated that 22,000 thrombosis related to COC use occur each year in Europe (McDaid et al., 2017). A major contributing factor is the presence of unknown coagulation abnormalities during contraceptive use. One of the most common inherited thrombophilia in Europe is the Factor V Leiden (FVL) mutation. The prevalence is about 5% in the Caucasian population and is present in 20% of patients suffering from VTE (McDaid et al., 2017). Other inherited clotting defects (i.e., prothrombin (PT) G20210A mutation, antithrombin, protein C or protein S deficiency) also increase the risk of VTE among COC users and account for an additional 2-3% in the general population (Douxfils, Morimont, & Bouvy, 2020; Hugon-Rodin et al., 2018). No systematic screening is, however, recommended at the time of the first prescription of a COC for different reasons including economic ones. Nevertheless, the presence of a known hereditary thrombophilia is contraindicated to COC use. As the standard of care, the suspicion of an underlying thrombophilia is based on familial history of VTE. This strategy is recognized for its low sensitivity and poor predictive value to identify patients with thrombophilia (Suchon et al., 2016). Consequently, there is a need of being able of identifying hereditary thrombophilia in women before introducing hormonal contraceptives. High responsiveness to oestrogenic compounds should also be pinned down. Such technology should be accessible at an acceptable price for the society to provide a cost-effective, but clinically mandatory, solution for this population of young and healthy women.

As the field of personalized medicines is currently expanding, identifying one or several biomarkers to establish a « coagulability status » would be an interesting

approach to minimize the risk of VTE in women on hormonal therapy. Activated protein C (APCR) could be one of the potential candidates. An acquired APCR is frequently observed in women during their lifetime and is related to the endogenous or exogenous hormonal changes including the use of hormonal contraception during the fertile period, the pregnancy and postpartum periods, or the use of menopausal replacement therapy during menopause.

Several methods for the detection of APCR have been developed but the ETP-based APCR assay is the most suitable for the assessment of COC-induced changes in haemostasis (Douxfils, Morimont, Delvigne, et al., 2020). The test is part of the European Medicines Agency's guidance for the proper assessment of the thrombogenic profile of new COC preparations (EMEA/CPMP/EWP/519/98 Rev 1). However, the absence of a standardized methodology clearly led to misinterpretation of the impact of the different generations of COC on APCR. For example, on previous studies, no significant difference between nonusers and women using 2nd generation COC (e.g. ethinylestradiol (EE) + levonorgestrel) was observed while other authors reported an increase of more than 150% of the normalized APC sensitivity ratio (nAPCsr) (Douxfils, Morimont, & Bouvy, 2020). In the study that used a less sensitive methodology, the mean nAPCsr was 0.70 in nonuser group and 0.78 in 2nd generation COC group while the reference population should have a nAPCsr around 1.0. This reveals that the methodology was not optimal since the reference plasma was more resistant than the patient's samples. In comparison, with the use of an ETP-based APCR assay developed, validated and standardized according to regulatory standards, the nAPCsr (normal range: 0.00-2.08) (Douxfils, Morimont, Delvigne, et al., 2020) raised at 4.09 in women using EE 20µg + levonorgestrel 100µg and 5.40 in women using EE 30µg/desogestrel 150µg. This support the assumption that an increased ratio may be correlated with the risk of VTE (Erreur ! Source du renvoi introuvable.) (de Bastos et al., 2014; Tans et al., 2003). These preliminary data support the concept that the nAPCsr, obtained with a test that uses a validated and standardized procedure with an appropriate sensitivity, could be a promising regulatory and clinical tool to identify the thrombogenicity of COCs (Douxfils, Morimont, & Bouvy, 2020). In addition, this would represent a more appropriate and cheaper alternative than a thrombophilia tests panel for assessing the risk since, this test does not only focus on the inherited coagulopathies but also assesses the individual sensitivity towards hormonal contraceptive preparations (Douxfils, Morimont, Delvigne, et al., 2020).

In the landscape of contraception pills, new compounds with safer haemostatic profile have also been proposed during the last 15 years. In their review, Abou-Ismail *et al.* discussed the oestradiol (valerate) containing pills but did not consider estetrol (E4), a native oestrogen produced by the human foetal liver and chemically

synthesized from oestrone. This new oestrogenic compound is currently proposed in association with drospirenone as a new COC. This combination showed a promising haemostatic profile which looks even safer than EE/levonorgestrel containing pills. The E4/drospirenone combination showed a reduced impact on several haemostasis parameters, including the prothrombin fragment 1+2, protein S and nAPCsr, as reported on **Figure II.1.1**. Furthermore, it also has less impact on the sex hormone-binding globulin (SHBG), a biomarker which reflects the total estrogenicity of a preparation and which has also been proposed to assess the VTE risk, although it is insensitive towards inherited coagulopathies. Results of the clinical program development of E4 clearly demonstrates that the oestrogenic moiety of a COC preparation is the sole responsible of the thrombogenicity, the progestin being only a "counterweight" thanks to its androgenic property (Douxfils, Klipping, et al., 2020).

To exemplify the prediction capacities of the nAPCsr, we did in silico modelling by combining both the nAPCsr for specific COC preparations with their respective VTE relative risk. The combination E4/drospirenone might express a relative risk [95%CI] of 1.29 [0.62-1.97]. Caution is required when interpreting these data since this prediction model is only exploratory and further investigations and validation are needed. However, these data support the idea that the nAPCsr could become a universal test to assess the hormone-induced risk of VTE in women during their entire lifetime. First, the assessment of the baseline risk will provide objective data for the gynaecologist to support the prescription of the more adapted hormonal therapy and, in case of high coagulability state, to suspect an underlying pathology and refer to a haematologist. Subsequently, testing after a sequence of 2 to 3 cycles of hormonal treatment will allow identifying women with an abnormal rise of the nAPCsr which may reveal an over-sensitivity of the oestrogenic effect of COC.

Additionally, information on the "coagulability status" could not only reduce the risk of COC-induced thrombosis but also the incidence of thrombotic events in situations associated with elevated thrombotic risk. Indeed, such a strategy could benefit to other therapeutic areas and may guide clinical decision is various settings.

Venous thromboembolism and pulmonary embolism are associated with significant mortality but also with a high morbidity rate leading to expensive costs for the diagnosis, the treatment, and the management of any thrombotic related disability, i.e., recurrent VTE, post-thrombotic syndrome, or chronic pulmonary hypertension.

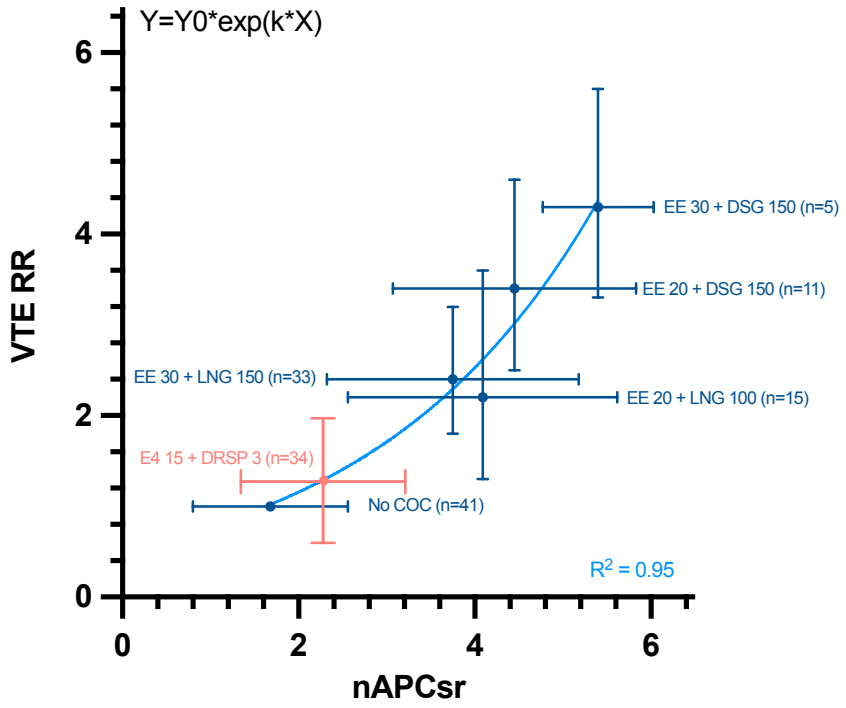

Figure II.1.1 | Correlation between nAPCsr and the relative risk of VTE (de Bastos, 2014) depending on the type of COC (i.e. (EE) 20 µg/levonorgestrel (LNG) 100 µg; EE 30 µg/LNG 150 µg; EE 20 µg/desogestrel (DSG) 150 µg; EE 30 µg/DSG 150 µg)
Nonuser group used as a reference (VTE RR=1) showed mean nAPCsr ± standard deviation of 1.68 ± 0.88 (n=41). EE 20/LNG 100 showed mean nAPCsr ± standard deviation of 4.09 ± 1.53 (n=15) and VTE RR (CI 95%) of 2.2 (1.3-3.6); EE 30/LNG 150 showed mean nAPCsr ± standard deviation of 3.75 ± 1.43 (n=33) and VTE RR (CI 95%) of 2.4 (1.8-3.2); EE 20/DSG 150 showed mean nAPCsr ± standard deviation of 4.45 ± 1.38 (n=11) and VTE RR (CI 95%) of 3.4 (2.5-4.6); EE 30/DSG 150 showed mean nAPCsr ± standard deviation of 5.40 ± 0.63 (n=5) and VTE RR (CI 95%) of 4.3 (3.3-5.6). New combination E4/DRSP showed a nAPCsr of 2.28 and an interpolated VTE RR (CI 95%) of 1.29 (0.62-1.97).

All of these led to an important financial burden within personal expenses, healthcare resources and societal costs (Vernon et al., 2017). Today, a complete thrombophilia assessment requires several coagulation assays which can make the interpretation of the results expensive and challenging. Studies assessing the cost effectiveness of thrombophilia screening prior prescribing COC are scarce and suffer from limitations (Douxfils, Klipping, et al., 2020). They usually do not take into account the long-term use of COC, the potential long-term consequences of VTE and above all, the lifetime benefit of screening, i.e., the awareness of inherited thrombophilia, the risk minimization strategies to reduced risk of VTE and the decreased economic burden (Vernon et al., 2017).

The ETP-based APCR assay, a thrombin generation based global assay, represents an interesting approach that needs further clinical validation to assess and score a hypercoagulable state that would help the clinician in decision-making. Finally, it would provide a simple, easy-to-implement and reassuring risk minimization measure for the safe use of COC and other hormone-related therapies and their derivatives.

Under submission

II.2 - PREDICTION OF VENOUS THROMBOEMBOLISM RISK ASSOCIATED WITH COMBINED ORAL CONTRACEPTIVES: AN EXPLORATORY MODEL

Laure Morimont, Mitchell D Creinin, Ulysse Gaspard, Jean-Michel Dogné, Jean-Michel Foidart and Jonathan Douxfils

SUMMARY

Introduction: Combined oral contraceptives (COCs) containing ethinylestradiol (EE) increase venous thromboembolism (VTE) risk compared to nonusers. COCs with oestradiol (E2) and estetrol (E4) have been introduced to lower this risk. Activated protein C resistance (APCR), a dysregulation of haemostasis, is recognized as a risk factor for VTE in COC users. The endogenous thrombin potential (ETP)-based APCR assay is the most suitable assessment of COC-induced coagulation changes.

Aim: We aimed to model the correlation between ETP-based APCR outcomes (expressed as normalized activated protein C sensitivity ratio (nAPCsr)) in users of COCs and VTE relative risk defined by large epidemiological studies.

Methods: We collected nAPCsr data for 332 specimens from non-COC users and from users of multiple COCs. Specimens came from the registered biobank of the University of Namur and from a recent phase II clinical trial. We stratified specimens according to COC use and we computed mean nAPCsr for each subgroup. We created models by associating these nAPCsr values with established VTE-associated relative risks (RRs) issued from epidemiological studies. We measured the strength of the association using goodness-of-fit and Spearman's rank correlation testing. We used the model to estimate VTE RR based on nAPCsr values for EE 30 µg/dienogest (n=14), E2/nomegestrol acetate (NOMAC) (n=7) and E4/drospirenone (n=32) users.

Results: nAPCsr data from nonuser women (n=162) and COC users (EE with levonorgestrel (EE 20 µg, n=28; EE 30 µg, n=36), desogestrel (EE 20 µg, n=37; EE 30 µg; n=7), with gestodene (EE 20 µg, n=7) and cyproterone acetate (EE=35 µg, n=3)) represented x-coordinates of our models. VTE RR associated with these COCs and extracted from de Bastos meta-analysis or Lidegaard study represented y-coordinates of our models. These 2 models provided exponential growth equations with excellent goodness-of-fit (R^2=.98 and .97) and Spearman's rank correlation (Rs=1.00). Based on these models, RR estimates were 3.70 or 3.74 for EE/dienogest, 1.68 for E2/NOMAC and 1.54 or 1.53 for E4/drospirenone versus non-COC users.

Conclusion: These exploratory models are strongly correlated with predicted RR estimates from published post marketing surveillance data comparing EE/dienogest

and E2/NOMAC VTE risk versus nonusers. The low predicted risk for E4/drospirenone fits with clinical studies results showing its low impact on haemostasis. These findings support that ETP-based APCR could become a surrogate biomarker for estimating the VTE risk of a particular COC, which represents the main cause of APCR in a young population.

INTRODUCTION

Combined hormonal contraceptives containing ethinylestradiol (EE) increase venous thromboembolism (VTE) risk compared to nonusers. The risk varies from 2.2-6.6 times and depends on the oestrogen doses and progestin components as well as clinical characteristics like Body Mass Index (BMI), diabetes, mellitus, hypertension, or polycystic ovary syndrome (de Bastos et al., 2014; Lidegaard et al., 2011; Vinogradova et al., 2015; Waddington et al., 2017). New combined oral contraceptives (COCs) with natural oestrogens, oestradiol (E2) and estetrol (E4), have been introduced in the last decade with the hope to significantly lower the VTE risk as compared to EE-containing regimens. To this point, no surrogate markers have been clearly identified that directly correlate with VTE risk in COC users. As such, we must wait for results of population-based phase IV trials which take several years to complete. For example, investigators reported, in 2021, results from a post-marketing study of E2 1.5 mg/nomegestrol acetate 2.5 mg, demonstrating a similar VTE risk compared to EE/levonorgestrel regimens (adjusted hazard ratio [HR] 0.59; 95% CI 0.25-1.35) more than a decade after the E2/nomegestrol product was first marketed (Reed et al., 2021). More recently, E4 15mg/drospirenone 3 mg has been introduced with phase II data demonstrating an almost neutral haemostatic profile (Douxfils, Klipping, et al., 2020; Douxfils, Morimont, et al., 2022); however, it will again be many years before phase IV epidemiological data are available.

Published data has confirmed activated protein C resistance (APCR), a dysregulation of haemostasis, as an independent VTE risk factor (de Visser et al., 1999; Rodeghiero et al., 1999). Several methods for APCR detection have been developed; however, the endogenous thrombin potential (ETP)-based APCR assay, developed in 1997, has proven to be the most suitable for the assessment of COC-induced changes on the coagulation system (de Visser et al., 2005; Douxfils, Morimont, & Bouvy, 2020; Morimont, Dogné, et al., 2020; Morimont, Donis, et al., 2022; Morimont, Haguet, et al., 2021). This test is required by the European Medicines Agency (EMA) as part of the haematological assessment of the thrombogenic profile of new COC preparations.(European Medicines Agency, 2005) However, the ETP-based APCR test suffered from a lack of standardization which hampered study-to-study comparison but recently a validated methodology (described in **Part I.1**) has been developed with the aim to propose a standardized and harmonized scale for the ETP-based APCR, the normalized activated protein C sensitivity ratio (nAPCsr).

In this report, we aim to evaluate nAPCsr data of users of multiple COCs and to correlate these data with the VTE risk observed in epidemiological studies as suggested in the previous chapter (i.e., **Part II.1**).

MATERIAL AND METHODS

This report is divided into 2 parts; first, we assessed ETP-based APCR of 332 existing plasma samples, using the validated methodology (**Part I.1**). In a second step, we created VTE predictive models using these nAPCsr data.

These existing plasma samples came from two sources, a recent phase II clinical trial (NCT 02957630) (n=153) and the Namur Biobank-eXchange (NAB-X), the registered biobank from the University of Namur (Namur, Belgium) (n=179).

The NAB-X specimens came from blood campaigns organized by the Department of Pharmacy at the University of Namur. These campaigns recruit volunteers to donate blood for research testing as approved by the Centre Hospitalier Universitaire, Université Catholique de Louvain (CHU UCL) Namur Ethical Committee. Written informed consent was obtained from each donor. At the time of collection, study staff collected demographics, medical history, and current medication use. They only allowed donation if volunteers do not have a history of thrombotic and/or haemorrhagic events, treatment by antiplatelets or anticoagulant medications or other drugs potentially affecting platelets or coagulation and pregnancy. After collection, NAB-X tested all specimens for the Factor V Leiden (FVL) and prothrombin (PT) G20210A mutations using a CE-in vitro diagnostic approved technique. Blood was collected in sodium citrates and processed to plasma before being frozen in liquid nitrogen and stored at ≤-70°C within the NAB-X biobank.

The phase II clinical trial from which we obtained specimen is the E4/DRSP Endocrine Function, Metabolic Control and Haemostasis Study (NCT 02957630). Inclusion and exclusion criteria have been described previously (Douxfils, Klipping, et al., 2020) but they will also be reported in the next section (**Part III**) which will be devoted to E4.

For this research project, we requested specimens from NAB-X with the following criteria: plasma from women of childbearing age (18-40 years), without any hormonal contraceptive method or who used the same COC for at least 3 cycles, and not carriers of FVL or PT G20210A mutations. These specimens had to be stored at ≤-70°C for less than 3 years, as plasma stability for the ETP-based APC-R assay is 36 months (**Part I.1**).

As for specimens from the phase II clinical trial, we only collected data from women who were under 40 years of age, at baseline and after 3 cycles of treatment (E4 15mg/drospirenone 3mg and EE 30µg/levonorgestrel 150µg).

For each specimen, the ETP-based APCR assay was performed as illustrated in the **Memento**.

To build VTE predictive models, we defined x-coordinates as mean nAPCsr values obtained in non-COC users and in users of different COCs. Y-coordinates

corresponded to VTE RRs (extracted from epidemiological studies) associated with the use of these same COCs versus nonusers. The correlation between nAPCsr and VTE RR was assessed with a Spearman rank correlation (Rs) and the goodness-of-fit was evaluated based on R squared (R^2).

Statistical analysis

Statistical analyses were performed using GraphPad version 9.3.1 (GraphPad Prism 9.3.1 for MacOs, GraphPad Software, San Diego, USA, www.graphpad.com). No formal sample size calculation was performed, and no formal statistical analysis was planned for this exploratory study.

RESULTS

Based on the collected 332 specimens, nonusers were pulled together, and COC-users were stratified according to the oestroprogestin association. Demographics of each subgroup are presented in **Table II.2.1** and mean ±SD nAPCsr values are reported in **Figure II.2.1**.

Table II.2.1 | Demographic data of collected specimens.

	Number of subjects	Mean age (ranges), yrs.	Mean BMI (range) kg/m²	Source of the data
Nonusers	162	23 (18-29)	22.6 (16.9-34.9)	NAB-X Sponsored trial
EE 20µg + levonorgestrel 100µg	28	21 (18-28)	22.4 (17.6-28.7)	NAB-X
EE 30µg + levonorgestrel 150µg	36	24 (18-39)	22.5 (17.4-29.8)	NAB-X Sponsored trial
EE 20µg + desogestrel 150µg	37	21 (18-29)	22.0 (17.7-31.3)	NAB-X
EE 30µg + desogestrel 150µg	7	22 (19-26)	21.8 (18.3-24.8)	NAB-X
EE 35µg + cyproterone acetate 2mg	3	20 (18-22)	22.8 (20.7-25.8)	NAB-X
EE 20µg + gestodene 75µg	6	22 (19-26)	21.1 (18.7-25.0	NAB-X
EE 30µg + dienogest 2mg	14	21 (19-27)	20.6 (18.5-25.1)	NAB-X
E2 1.5mg + nomegestrol acetate 2.5mg	7	21 (18-25)	21.5 (16.7-25.0)	NAB-X
E4 15mg + drospirenone 3mg	32	25 (19-36)	23.2 (19.2-28.7)	Sponsored trial

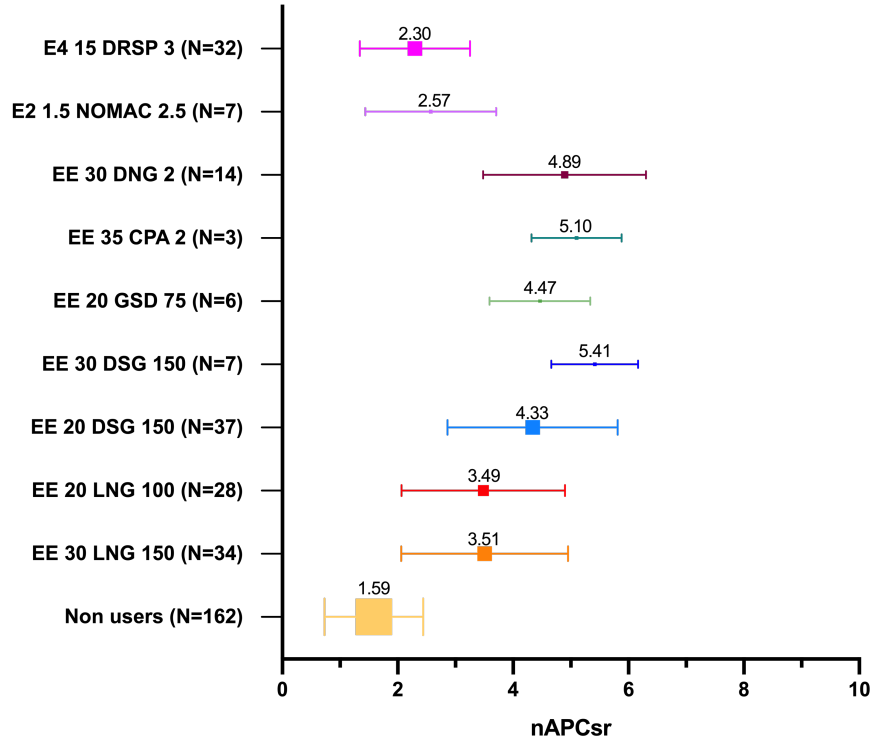

Figure II.2.1 | nAPCsr of nonuser women and users of COCs.
Means ± standard deviations are represented. The size of the square is proportional to the number of subjects included in each subgroup.

We created 2 predictive models by using 2 different sources for the extraction of VTE RR associated with COCs, namely the meta-analysis of *de Bastos* (de Bastos et al., 2014) and the Danish cohort study of *Lidegaard* (Lidegaard et al., 2011).

For the model based on *de Bastos'* meta-analysis, we used nAPCsr results of the following subgroups: nonusers and users of EE 20µg/levonorgestrel 100µg; EE 30µg/levonorgestrel 150µg; EE 20µg/desogestrel 150µg; EE 30µg/desogestrel 150µg; and EE 35µg/cyproterone acetate 2mg. The nonuser group was used as the comparator with a VTE RR of 1.0. For *Lidegaard's* model, included COCs slightly differed compared to *de Bastos'* model. Nonusers remained the comparator group and included COCs were EE 30µg/levonorgestrel 150µg; EE 20µg/desogestrel 150µg; EE 30µg/desogestrel 150µg; EE 20µg/gestodene 75µg and EE 35µg/cyproterone acetate 2mg. (x; y) coordinates, i.e. (mean nAPCsr; extracted VTE RR) for each model are shown in

Table II.2.2. The mathematical formula coming from our models was y=Y0*exp(k*X), representing an exponential growth equation.

Table II.2.2 | (x; y) coordinates corresponding to (mean nAPCsr; extracted VTE RR) to build prediction models.

	X-coordinate (mean nAPCsr ±SD)	**Y-coordinate**	
		VTE RR ±95%CI extracted from *de Bastos'* meta-analysis	VTE RR ±95%CI extracted from *Lidegaard's* study
Nonusers	1.59 ±0.86	1	1
EE 20µg + levonorgestrel 100µg	3.49 ±1.42	2.2 (1.3-3.6)	2.19 (1.74-2.75)
EE 30µg + levonorgestrel 150µg	3.51 ±1.45	2.4 (1.8-3.2)	-
EE 20µg + desogestrel 150µg	4.34 ±1.47	3.4 (2.5-4.6)	3.26 (2.88-3.69)
EE 30µg + desogestrel 150µg	5.41 ±0.75	4.3 (3.3-5.6)	4.21 (3.63-4.87)
EE 35µg + cyproterone acetate 2mg	5.10 ±0.78	3.9 (2.7-5.5)	4.10 (3.37-4.99)
EE 20µg + gestodene 75µg	4.47 ±0.87	-	3.50 (3.09-3.97)

As shown in **Figure II.2.2**, both models presented excellent goodness of fit (R^2 0.98, de Bastos; R^2 0.97, Lidegaard) and a strong correlation between nAPCsr and VTE risk (Rs 1.000 for both).

We used the models to estimate VTE RR of oestroprogestin associations for which we had collected nAPCsr values, but whose risk had not been determined in the meta-analysis of *de Bastos* or the study of *Lidegaard*. Three COCs were concerned; EE 30µg/dienogest 2mg (n=14); E2 1.5mg/nomegestrol acetate 2.5mg and E4 15mg/drospirenone 3mg and estimates are shown in **Figure II.2.2** and **Table II.2.3.**

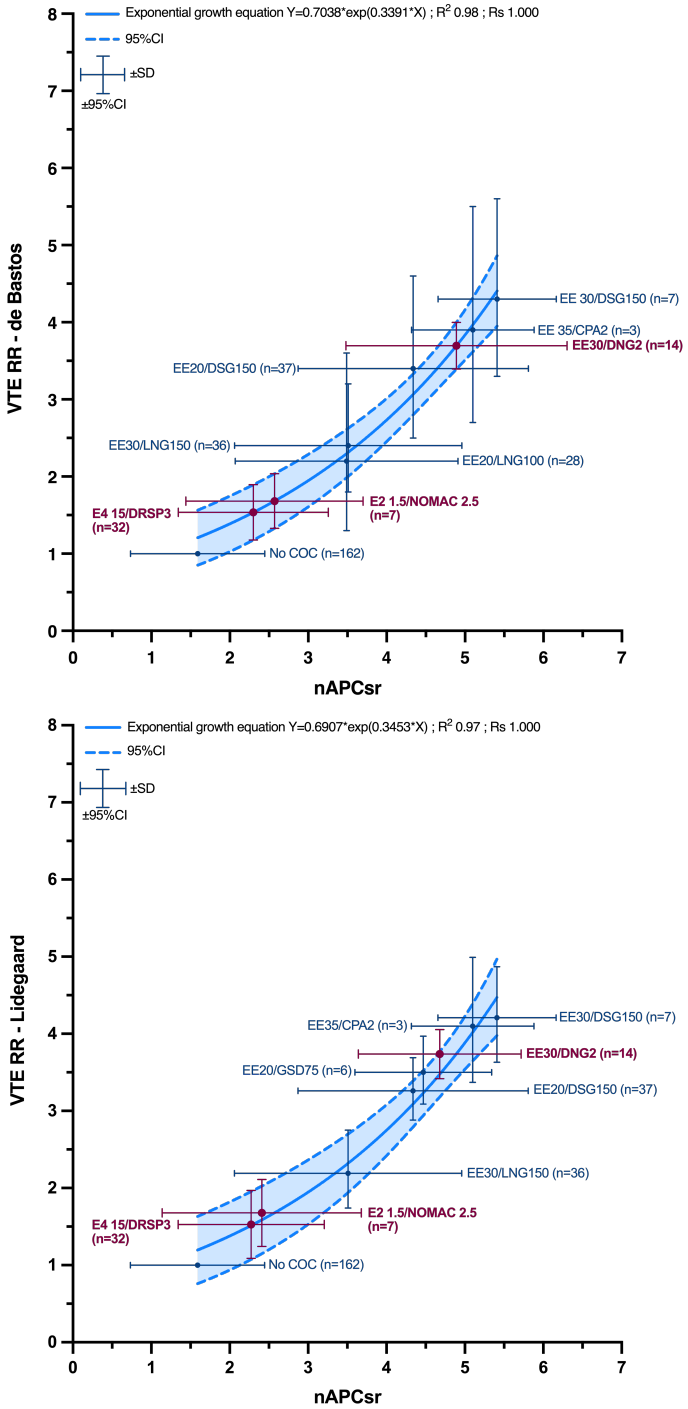

Figure II.2.2 | VTE prediction exploratory model based on *de Bastos'* meta-analysis (upper graph) and *Lidegaard's* study (lower graph).

Table II.2.3 | VTE risk estimates determined by interpolations on the prediction VTE models.

	X-coordinate (mean nAPCsr ±SD)	**Interpolated Y-coordinate**	
		VTE RR ± 95%CI based on *de Bastos'* meta-analysis	VTE RR ± 95%CI based on *Lidegaard's* study
EE 30µg + dienogest 2mg	4.89 ±1.41	3.70 (3.39-4.00)	3.74 (3.42-4.06)
E2 1.5mg + nomegestrol acetate 2.5mg	2.57 ±1.13	1.68 (1.33-2.04)	1.68 (1.24-2.11)
E4 15mg + drospirenone 3mg	2.30 ±0.96	1.54 (1.18-1.89)	1.53 (1.09-1.97)

DISCUSSION

This research project has shown that nAPCsr values may reflect the thrombotic risk profile of COCs.

Based on 162 specimens from nonuser women, the mean nAPCsr equalled 1.59 which stands within the normal range of 0.00 to 2.08 (Morimont, Didembourg, et al., 2021). Regarding EE-based COC preparations, mean nAPCsr were out of the normal range, with different levels of APCR depending on the association. The lowest impact was for levonorgestrel-containing products with a mean nAPCsr of 3.49 and 3.51 for EE 20µg/levonorgestrel 100µg and EE 30µg/levonorgestrel 150µg, respectively. As already discussed in the introductory part, this less pronounced impact on APCR compared to the other preparations could be explained by the strong androgenic properties of levonorgestrel, allowing counterbalancing, to a certain degree, the procoagulant effect induced by EE (Kuhl, 2011; Lawrie et al., 2011; Morimont, Haguet, et al., 2021; Regidor, 2018). Furthermore, whether 20 or 30µg of EE were used, APCR remained similar, as the levonorgestrel dosage is proportionally increased (i.e., 150µg with EE 30µg versus 100µg with EE 20µg; 1:5 ratio). Regarding desogestrel-containing products, the low EE-dosage led to a mean nAPCsr of 4.34 while the high EE-dosage led to a mean nAPCsr of 5.41. As desogestrel shows weak androgenic properties, it is indeed less prone to offset the negative impact of EE on haemostasis, resulting in a higher level of APCR compared to levonorgestrel-containing products. Furthermore, as the dosage of the progestin remains the same whether 20 or 30µg of EE is used, APCR is more pronounced with the high EE dosage. For preparations containing EE associated either with gestodene or cyproterone acetate, APCR levels were of the same order as desogestrel-based products as the associated progestin also demonstrate weak androgenic properties and even anti-androgenic ones (e.g., cyproterone acetate)(Morimont, Haguet, et al., 2021). These clinical laboratory data are in line with epidemiological data, in which the lowest risk

of VTE was associated with levonorgestrel-containing products while the highest was observed with 30 or 35µg of EE associated with desogestrel or cyproterone acetate (de Bastos et al., 2014; Lidegaard et al., 2011; Vinogradova et al., 2015).

For COCs that were not part of the model but for which epidemiological studies have been performed after the release of *de Bastos* or *Lidegaard's* data, i.e., EE 30µg/dienogest 2mg and E2 1.5mg/nomegestrol acetate 2.5mg, VTE RR estimated by the models were similar to those reported in their COC-specific epidemiological studies (Dinger, 2020; Reed et al., 2021). Based on the mean nAPCsr (±SD) we calculated, i.e., 4.89 (±1.41) for EE 30µg/dienogest 2mg, the RR of VTE was 3.70 and 3.74 depending on the model used. In the study of Dinger published in 2020 (Dinger, 2020) the calculated RR of VTE compared to nonusers was around 3.4, an estimate which is in line with the estimated RR provided by the models. For E2 1.5mg/ nomegestrol acetate 2.5mg, mean nAPCsr value (±SD) equalled 2.57 ± 1.13, which is translated into an estimated RR of VTE of 1.68 versus nonusers. This is again in line with results of the PRO-E2 study which reported a RR of VTE around 1.6 for E2 1.5mg/ nomegestrol acetate 2.5mg compared to nonusers (Reed et al., 2021). These external validations permit us to appreciate the robustness of the model. We suggest that it could be used to fairly estimate the RR of VTE of COC preparations for which the risk is currently unknown due to the absence of sufficient epidemiological data. The risk of VTE for E4 15mg/DRSP 3mg has not been yet established by an epidemiological study. The models described here permit to hypothesize that the VTE RR is around 1.60 compared to nonusers (**Figure II.2.2**)**.** This risk estimate is not different from that of the E2/nomegestrol acetate users but lower than all other COC users, which is in line with the current knowledge about this new COC. Indeed, according to data from the phase II clinical trials and the exhaustive evaluation of the impact of this COC on haemostasis (which will be described in **Section III**), the risk of VTE is expected to be low with this preparation.(Douxfils, Klipping, et al., 2020; Gerard et al., 2022)

One of the study limitations of these exploratory models is the small number of women evaluated in each subgroup. These models remain to be validated in larger cohorts. According to the Clinical and Laboratory Standards Institute on the definition of the laboratory reference intervals (CLSI EP28-A3C document (Clinical and Laboratory Standards Institute, 2010)), at least 120 values are required for the establishment of a reference range for a particular biological parameter and the nAPCsr reference range should be established for each COC preparation.

Although the nested case-control study of *Vinogradova's* (Vinogradova et al., 2015) provided more recent data compared to *Lidegaard's* or *de Bastos*', the lack of stratification by EE dosage does not permit to build a model using these epidemiological data. Therefore, an updated network meta-analysis would be of

great interest to include new COC preparations available on the market, e.g., E2-containing products and to obtain consistent data based on a pharmacodynamic classification of COCs. Nevertheless, the current models show reassuring robustness which to reliably support and validate the computation process of nAPCsr data to estimate the risk of VTE of a particular COC.

CONCLUSION

In definitive, these in silico modelling could finally serve for regulatory purpose since it could permit to avoid waiting almost a decade before positioning on the potential risk of VTE of a particular COC. It may also save time and money for the pharmaceutical companies and the regulators and may facilitate the uptake of innovative compounds for the clinicians by providing reassuring data on the risk of VTE with hormonal therapies. After validation, this model could reveal useful for the individual assessment of the VTE risk in any individual prescribed a COC.

PART III: ESTETROL ASSOCIATED WITH DROSPIRENONE – GLOBAL ASSESSMENT OF THE COAGULATION THROUGH THROMBIN GENERATION

III.1 - EVALUATION OF HAEMOSTASIS PARAMETERS

Adapted from the Publication of Douxfils et al. (2020) "Evaluation of the Effect of a New Oral Contraceptive Containing Estetrol and Drospirenone on Haemostasis Parameters". Contraception, 102(6):396-402

In 2021, a combination of 15mg estetrol (E4) and 3mg drospirenone (Nextstellis® in the US, Drovelis® and Lydisilka® in Europe) has been approved as a new combined oral contraceptive (COC). Prior to approval, the haemostasis profile of this new association was investigated in the Estelle phase II trial "E4/DRSP Endocrine Function, Metabolic Control and Haemostasis study" (NCT02957630).

This section aims to present the main changes in haemostasis biomarkers induced by E4 15mg/drospirenone 3mg in comparison with ethinylestradiol (EE)-containing products.

Briefly, this was a randomized, single centre, open label, exploratory study in which healthy women received either E4 15mg/drospirenone 3mg, ethinylestradiol (EE) 30µg/levonorgestrel 150µg, or EE 20µg/drospirenone 3mg for six 28-day cycles. Blood was collected at baseline, cycle 3 and cycle 6.

The following coagulation and fibrinolytic parameters were investigated: fibrinogen, prothrombin, factor VII, factor VIII, von Willebrand factor, antithrombin, protein S activity, free protein S, protein C, free tissue factor pathway inhibitor (TFPI), plasminogen, plasminogen activator inhibitor type-1 (PAI-1), tissue plasminogen activator (tPA), normalized activated protein C sensitivity ratio (nAPCsr), D-dimer and prothrombin fragment 1+2. In addition, sex hormone-binding globulin (SHBG) was also measured. Relative median changes from the baseline were evaluated.

The per protocol haemostasis population included 88 subjects among which, 34 in the E4/drospirenone arm, 25 in the EE/levonorgestrel group and 29 in the EE/drospirenone group.

As shown in **Figure III.1.1**, after 6 cycles of treatment, no relevant changes were observed for FVII, FVIII, VWF, AT, protein S (free and activity), protein C, TFPI, PAI-1 and tPA levels with E4/drospirenone. Compared to EE/levonorgestrel and EE/drospirenone, E4/drospirenone had less pronounced effects on the percentage change from the baseline of the nAPCsr (+30% versus +165% and +219%), plasminogen (+12% versus +40% and +36%), tPA (-7% versus -33% and -40%) and prothrombin fragment 1+2 (+23% versus +71% and +64%). For factor VII, protein C, protein S activity, free-protein S and SHBG the changes observed for E4/drospirenone were comparable to those for EE/levonorgestrel but lower than those for EE/drospirenone. No differences were seen for fibrinogen, prothrombin,

factor VIII, von Willebrand factor, antithrombin, free TFPI, and PAI-1. Results were similar after 3 treatment cycles.

These results showed that changes in haemostasis parameters after treatment with 6 cycles of E4/drospirenone were smaller or similar to those observed for EE/levonorgestrel. Similar, but more pronounced changes were also observed versus EE/drospirenone, which supports the hypothesis that the effect of COCs on haemostasis parameters is mainly mediated by the oestrogenic component but also, the nature of the progestin compound comes into play and modulates the global estrogenicity of the contraceptive agent.

Nevertheless, the synergistic effect of these changes could not be captured by these singular measurements. Hence the aim of the following section, which discusses the impact of these treatments on a global coagulation test, i.e., the thrombin generation assay.

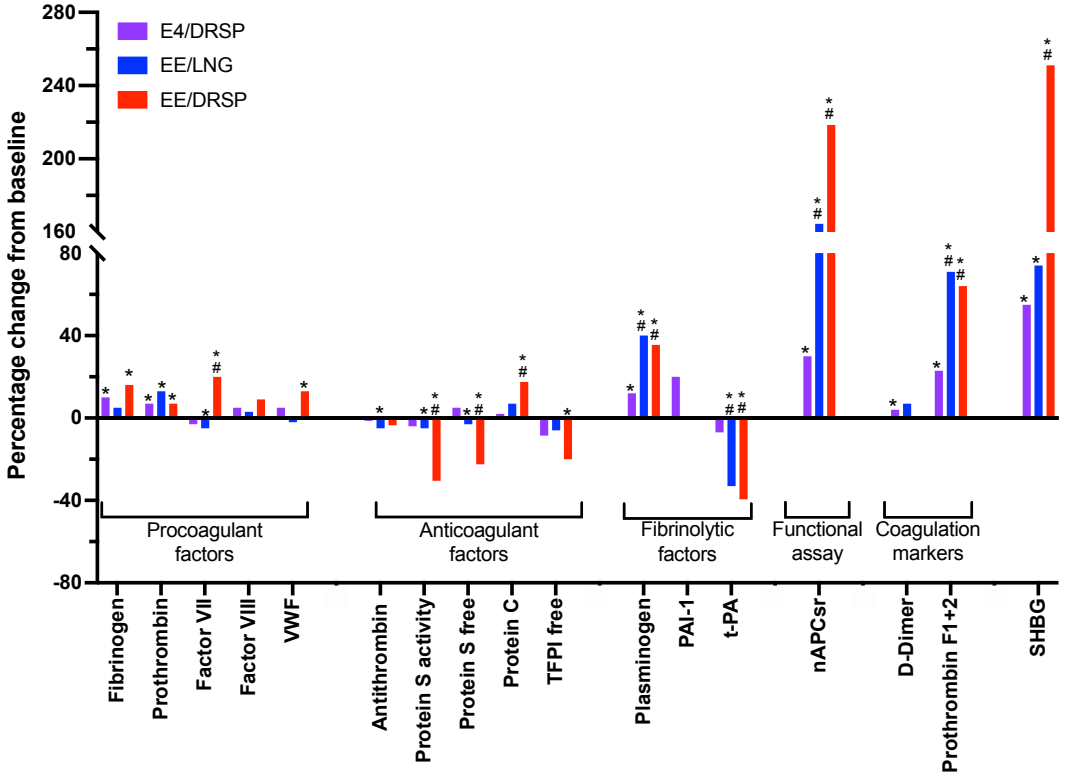

Figure III.1.1 | Effect of study treatment at cycle 6 on relevant haemostasis biomarkers and sex hormone-binding globulin (SHBG) – adapted from (Douxfils, Klipping, et al., 2020)

The columns represent the median of the percent change from the baseline (cycle 6 – baseline) - *different versus baseline, P<.05 using a signed rank test; # different from treatment with E4 15mg/DRSP 3mg, P<.05 using the Dwass-Steel-Critchlow-Fligner test.

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III.2 - Low Thrombin Generation in Users of a Contraceptive Containing Estetrol and Drospirenone

Laure Morimont, Maud Jost, Ulysse Gaspard, Jean-Michel Foidart, Jean-Michel Dogné, Jonathan Douxfils

Summary

Objective: To compare the impact on thrombin generation of the new combined oral contraceptive containing 15mg estetrol (E4) and 3mg drospirenone (DRSP) with ethinylestradiol (EE) (30 or 20µg) associated either with 150µg levonorgestrel (LNG) or with 3mg DRSP.

Methods: Data were collected from the "E4/DRSP Endocrine Function, Metabolic Control and Haemostasis Study" (NCT02957630). Overall, the per protocol set population included 24 subjects in the EE/LNG arm, 28 subjects in the EE/DRSP and 34 subjects in the E4/DRSP arm. Thrombograms and thrombin generation parameters (lag time; peak; time to peak; endogenous thrombin potential and mean velocity rate index) were extracted for each subject at baseline and after 6 cycles of treatment.

Results: After 6 cycles of treatment, EE-containing products arms show mean thrombograms outside the upper limit of the reference range, that is the 97.5th percentile of all baseline thrombograms. On the other hand, the mean thrombogram of E4/DRSP is within this reference interval. After 6 cycles of treatment, all thrombin generation parameters are statistically less impacted by E4/DRSP than EE-containing products.

Conclusions: In conclusion, an association of 15mg E4 with 3mg DRSP does not have an impact on thrombin generation compared to EE-containing products which, either associated with LNG or DRSP, are able to increase the production of procoagulant factors and decrease the production of anticoagulant ones, shifting the patient to a prothrombotic state. EE-containing products thus generate prothrombotic environments contrary to E4 which demonstrates a neutral profile on haemostasis.

INTRODUCTION

Pregnancy and postpartum, as well as exogenous hormones exposure, such as combined hormonal contraceptives (CHCs), create hormonal changes associated with an increased risk of venous thromboembolism (VTE) (Speed et al., 2018). Indeed, a 5-fold increased risk of VTE is reported during pregnancy, and up to a 20- to 60-fold increased risk in the postpartum period (i.e., during the first 6 weeks after delivery) (Heit et al., 2005; Jackson et al., 2011; Pomp et al., 2008). For women using CHCs, the relative risk varies between 1.3 and 5.6 depending on the oestroprogestin association and the dose of the oestrogenic component (de Bastos et al., 2014; Dinger et al., 2016; Jick et al., 1995; Lidegaard et al., 2009; Rovinski et al., 2018; Spitzer et al., 1996; van Hylckama Vlieg et al., 2009).

Pregnancy and the use of CHCs cause changes in plasma levels of almost all proteins involved in the coagulation and fibrinolysis (Douxfils, Morimont, & Bouvy, 2020). These changes might be considered as relatively modest when measured separately but they could have a supra-additive effect, as explained in the **introductory part**, leading to a procoagulable state responsible for this increased risk of VTE (Morimont, Haguet, et al., 2021). Overall, rises in coagulation factors II, V, VII, VIII, IX, X, XI, XII and von Willebrand factor (VWF), as well as fibrinogen levels are observed (Douxfils, Morimont, & Bouvy, 2020). On the other hand, antithrombin, protein S and tissue factor pathway inhibitor (TFPI) levels, three proteins contributing to the anticoagulant system, are decreased (Bonnar, 1987; Meade et al., 1977; Stadel, 1981; Wessler et al., 1976). As for the fibrinolysis, there is an increase in plasminogen levels but a decrease in tissue plasminogen activator (tPA) antigens and plasminogen activator inhibitor-1 (PAI-1) levels (Douxfils, Morimont, & Bouvy, 2020). These hormonal changes, both during pregnancy and following the use of hormonal therapy, are also associated with activated protein C (APCR) which can result from increases in FII, FVIII or FX levels and/or decreases in protein S and TFPI (Brenner, 2004; de Visser et al., 2005; Douxfils, Morimont, & Bouvy, 2020; Morimont, Haguet, et al., 2021).

Among assays measuring APCR, the endogenous thrombin potential (ETP)-based APCR assay is the most sensitive towards acquired APCR and has been linked to an increased risk of VTE in women on hormonal therapy (de Visser et al., 2005; Morimont, Dogné, et al., 2020; Morimont, Haguet, et al., 2021). This technique relies on the thrombin generation assay (TGA) which permits to obtain a thrombogram, i.e., a visual and quantitative representation of the amount of thrombin generated over time in a cupule. While the normalized APC sensitivity ratio (nAPCsr) reflects the capacity of the ETP-parameter (representing the area under the thrombogram) to be reduced in presence of exogenous APC, other parameters of the thrombogram can be exploited (Douxfils, Morimont, Delvigne, et al., 2020). Indeed, they can

provide information on the prothrombotic tendency (Lutsey et al., 2009; van Hylckama Vlieg et al., 2007) independently of the resistance towards exogenous APC. Besides, the use of ethinylestradiol (EE) based-CHCs, and other known hypercoagulable states, have been shown to enhance the in vitro thrombin generation (Hugon-Rodin et al., 2017; Mohamed et al., 2018; Tchaikovski et al., 2007).

A combination of 15mg estetrol (E4) and 3mg drospirenone (Nextstellis® in the US, Drovelis® and Lydisilka® in Europe) has recently been approved (Gerard et al., 2022). Estetrol is a natural and native foetal oestrogen synthesized exclusively in the human foetal liver (Holinka et al., 2008). It has a unique mode of action, different from those of other oestrogens, by activating the nuclear oestrogen receptor α (Erα) but antagonizing the membrane Erα (Abot et al., 2014). The use of E4 demonstrated a low impact on the liver with minimal effects on lipids, lipoproteins, sex hormone-binding globulin (SHBG) and several coagulation and fibrinolytic proteins (Douxfils, Klipping, et al., 2020). The association of E4 with drospirenone also showed a much lower impact on APCR compared to EE with levonorgestrel or EE with drospirenone as well as on the level of prothrombin fragment 1+2, a marker of the ongoing coagulation (Douxfils, Klipping, et al., 2020). Nevertheless, while some coagulation factors like prothrombin, FVII, TFPI or protein S were individually impacted by each of these therapies, the synergistic effect of these changes on the haemostasis could not be captured by these singular measurements. Therefore, a global test capable of capturing all pro- and anticoagulant factors level changes, would allow a more accurate evaluation of the impact of a CHC on haemostasis and the associated risk of VTE. The thrombin generation test permits to assess the coagulation process in its entirety, and it has been shown to be sensitive to the synergistic haemostatic alterations induced by CHCs (Hemker et al., 2003). This study aims therefore at comparing the impact of E4/drospirenone with EE/levonorgestrel and EE/drospirenone on thrombin generation.

MATERIAL AND METHODS

Study design

This single centre, randomized, open-label, controlled, three arms, parallel study in healthy females was conducted from September 2016 through October 2017 at Dinox BV, Groningen, the Netherlands (Eudra CT 2916-001316-37, Clinicaltrials.gov NCT02957630). The study, performed in accordance with the Declaration of Helsinki and the International Council for Harmonization (ICH) E6 (R2) Good Clinical Practice guidelines, was approved by an independent local ethics committee and written informed consent was obtained from all participants before study entry. The study consisted of a pre-treatment cycle (baseline), followed by six 28-day treatment

cycles. A total of 100 healthy women (40 in the investigational group and 30 per comparator group) was planned to be included in the study. Visits were planned to be at screening, at randomization/baseline, at cycle 3, at cycle 6 and/or at the end of the study. As haemostatic results at cycle 3 were similar to those of cycle 6, only data obtained at cycle 6 will be reported.

Study population

Healthy females aged 18-50 years with a body mass index (BMI) between 18 and 30 kg/m^2, and a natural menstrual cycle of maximum 35 days were eligible for inclusion. Main exclusion criteria were contraindications for the use of hormonal contraceptives, known coagulopathy or thrombogenic mutation, the use of anticoagulants or other drugs affecting coagulation and platelet aggregation and an abnormal Papanicolaou smear test. The use of an injectable contraceptive was not allowed within 3-10 months prior to the screening, depending on the type of injection. Women with CHC use prior to the study had a washout period of 4 weeks before pre-treatment cycle. Pre-treatment cycle started on the first day of the menstrual cycle (following the washout cycle for former CHC users). All subjects started the intake of the active study medication on the first day of their menstrual cycle following the pre-treatment cycle.

Study treatment

Eligible subjects were stratified by previous hormonal contraceptive use (8 weeks or >8 weeks without use before study treatment start) and by age (≤35 years or >35 years of age). Subjects were then assigned, using a computerized random allocation sequence, to one of the following treatments in a 4:3:3 ratio: 15mg E4 (as monohydrate, equivalent to 14.mg anhydrate) combined with 3 mg drospirenone (24-day active/4-day placebo regimen); 30µg EE combined with 150µg levonorgestrel (21-day active/7-day placebo regimen), or 20µg EE combined with 3mg drospirenone (24-day active/4-day placebo regimen). The E4/drospirenone-containing product was manufactured by Haupt Pharma, Münster, Germany and provided by Estetra SRL, an affiliate's company of Mithra Pharmaceuticals, Liège, Belgium. The other two products, i.e., EE/levonorgestrel (Melleva® 150/30, Leon Farma) and EE/drospirenone (Yaz®, Bayer Healthcare) were obtained from a local pharmacy. Study treatment started on the first day of the menstrual cycle following the pre-treatment cycle. Treatment compliance was verified using a diary and check of returned packages.

Study assessment and outcome parameters

Haemostasis parameters (fibrinogen, prothrombin, factor VII, FVIII, von Willebrand factor, antithrombin, protein S activity, free protein S, protein C, free TFPI, plasminogen, plasminogen activator inhibitor type-1 (PAI-1), tissue plasminogen

activator (tPA), ETP-based APCR (expressed as nAPCsr), D-dimers and prothrombin fragment 1+2 as well as SHBG have previously been reported (Douxfils, Klipping, et al., 2020) (**Part III.1**).

Thrombin generation assay has been performed on a Calibrated Automated Thrombogram (CAT) (Diagnostica Stago, Asnières-sur-Seine, France) using the STG-ThromboScreen® (Diagnostica Stago) as triggering reagent. A complete description of the method is illustrated in the **Memento.**

Statistical analysis

Statistical analysis was performed using GraphPad version 9.3.1 (GraphPad Prism 9.3.1 for MacOS, GraphPad Software, San Diego, California, USA, www.graphpad.com). All randomized subjects who received at least one dose of the study medication and had at least one haemostasis assessment on treatment, without any major protocol deviation impacting the endpoints, were included in the analysis (per protocol dataset). Descriptive statistics were used to analyse the data (n, mean, standard deviation [SD], median, minimum-maximum range, 10-90th percentile, and 95% confidence intervals [95%CI]). Changes of the different TGA parameters from the baseline to cycle 6 have been computed using paired t-tests and differences between treatment groups for a particular time point were assessed using an ordinary one-way ANOVA followed by a Tukey's multiple comparison test. Reference ranges for thrombin generation and associated parameters are reported as the 2.5th – 97.5th percentile of the entire baseline cohort (n=86), in accordance with the definition of the reference intervals as reported in the Clinical & Laboratory Standards Institute (CLSI) EP-28-A3C (Clinical and Laboratory Standards Institute, 2010). All statistical tests were evaluated with a level of significance of 0.05.

RESULTS

Study population

A total of 143 subjects were screened for eligibility of which 101 were randomized, and 98 received study treatment and 88 among these participants completed the study (per protocol set population) (**Figure III.2.1**). A summary of the demographic data at study entry is presented in **Table III.2.1** and shows no difference between groups at baseline. There was no important protocol deviation, including non-compliance issues. For one patient in the EE/drospirenone group, there was no sufficient plasma sample and thrombin generation could not be performed. One patient in the EE/levonorgestrel group with a thrombin generation curve at baseline defined as an outlier was not included in TGA analyses.

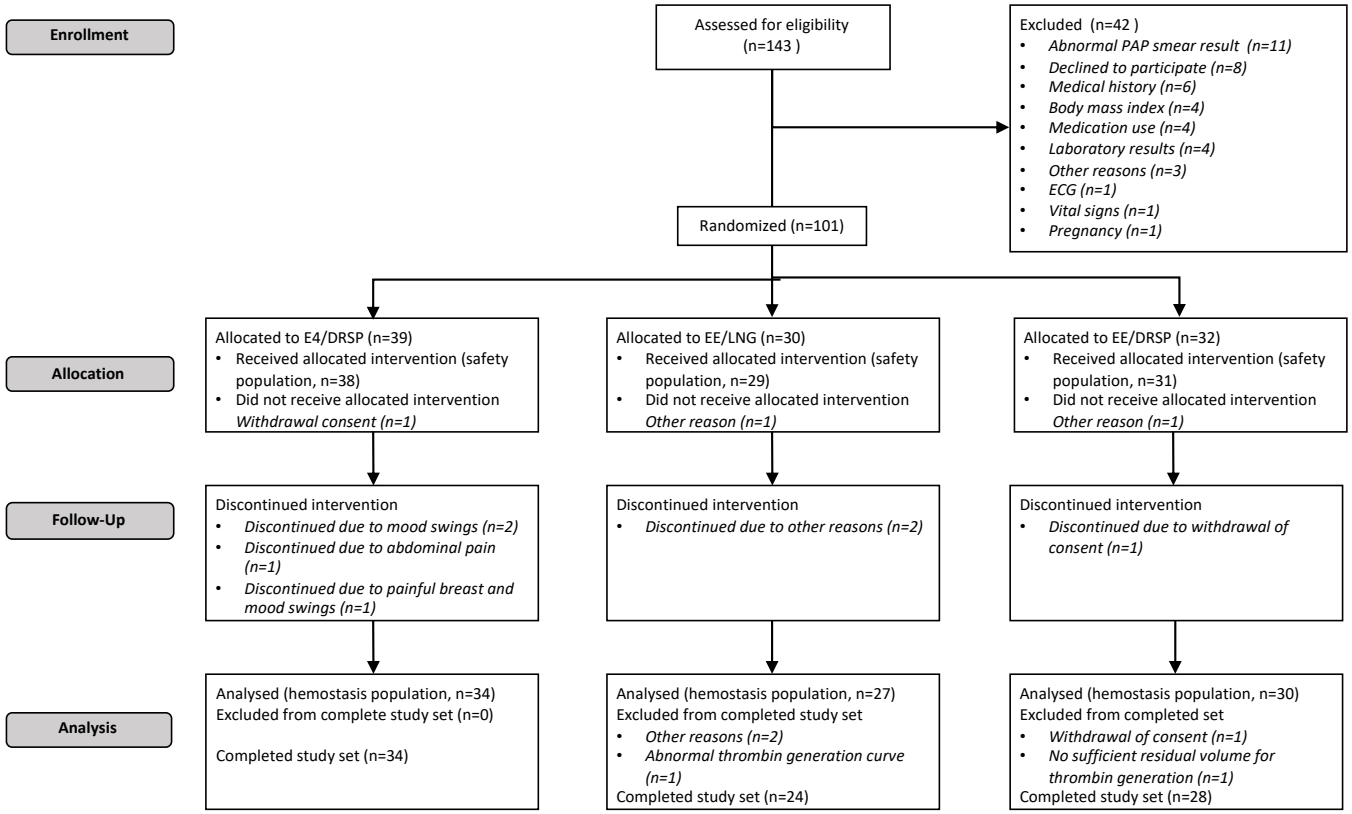


Figure III.2.1 | Trial flow diagram

The final per protocol set population for this study was therefore 86 among which 24 subjects received EE/levonorgestrel, 28 received EE/drospirenone and 34 received E4/drospirenone. Previous use of CHC concerned 38% of women in the EE/levonorgestrel group, 43% in the EE/drospirenone group, and 47% in the E4/drospirenone group.

Table III.2.1 | Mean demographic data at study entry.

	15mg E4 - 3mg DRSP (n=38)	**30µg EE - 150µg LNG (n=29)**	**20µg EE - 3mg DRSP (n=31)**	**All (n=98)**
Age, y (range)	26.7 (19 – 47)	26.2 (18 – 44)	25.6 (18 – 40)	26.2 (18 – 47)
Weight, kg (range)	68.1 (53.1 – 97.8)	65.6 (50.4 – 79.2)	63.2 (50.3 – 80.7)	65.8 (50.3 – 97.8)
Height, cm (range)	170.8 (159 – 188)	169.6 (160 – 181)	168.4 (155 – 183)	169.7 (155 – 188)
BMI, kg/m² (range)	23.33 (19.2 – 30.0)	22.83 (18.3 – 29.8)	22.27 (18.6 – 26.7)	22.85 (18.3 – 30.0)

Thrombogram and TGA parameters

Absolute values at baseline and after 6 cycles of treatment

Analyses of thrombograms and resulting TGA parameters were performed on data from the 86 subjects in the final per protocol set population.
Thrombograms at baseline (n=86, entire baseline cohort) and after 6 cycles of treatment of women either treated with EE/levonorgestrel (n=24), EE/drospirenone (n=28) or E4/drospirenone (n=34) are shown in **Figure III.2.2.**

The mean thrombogram along with the 2.5^{th}-97.5^{th} percentile is shown for the entire baseline cohort (n=86) and represents the reference interval. Mean [±95%CI of the mean] thrombograms after 6 cycles of treatment are also presented. EE/levonorgestrel and EE/drospirenone groups show mean thrombograms [±95%CI of the mean] outside the upper limit of the reference range, i.e., the 97.5^{th} percentile of all baseline thrombograms. On the other hand, the mean [±95%CI of the mean] thrombogram of E4/drospirenone is within this reference interval. Mean values of each TGA parameter at baseline and at cycle 6 of the different treatment arms are reported in **Table III.2.2.**

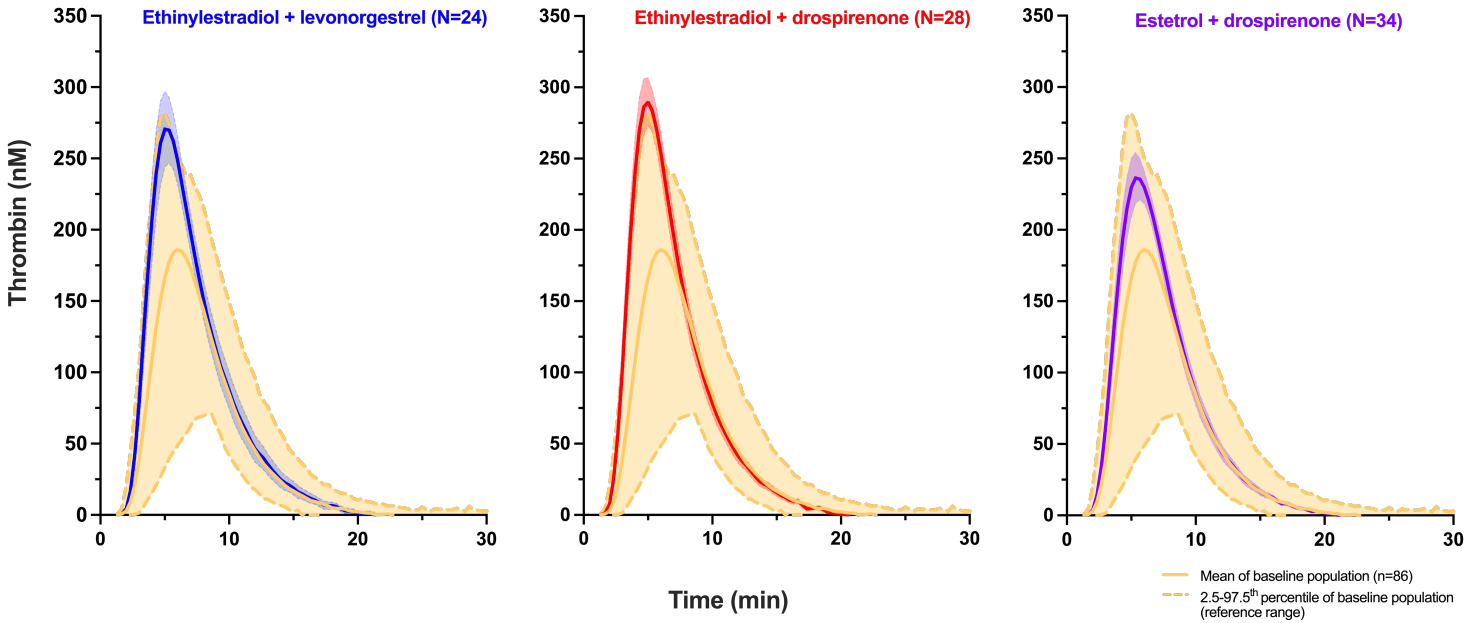


Figure III.2.2 | The mean (2.5th-97.5th percentiles) thrombograms of the entire baseline cohort (N=86) and mean thrombogram (95%CI of the mean) after 6 cycles of treatment are presented.

Table III.2.2 | Thrombin generation parameters with the different COC associations at baseline and cycle 6.
Mean values ± standard deviations are shown.

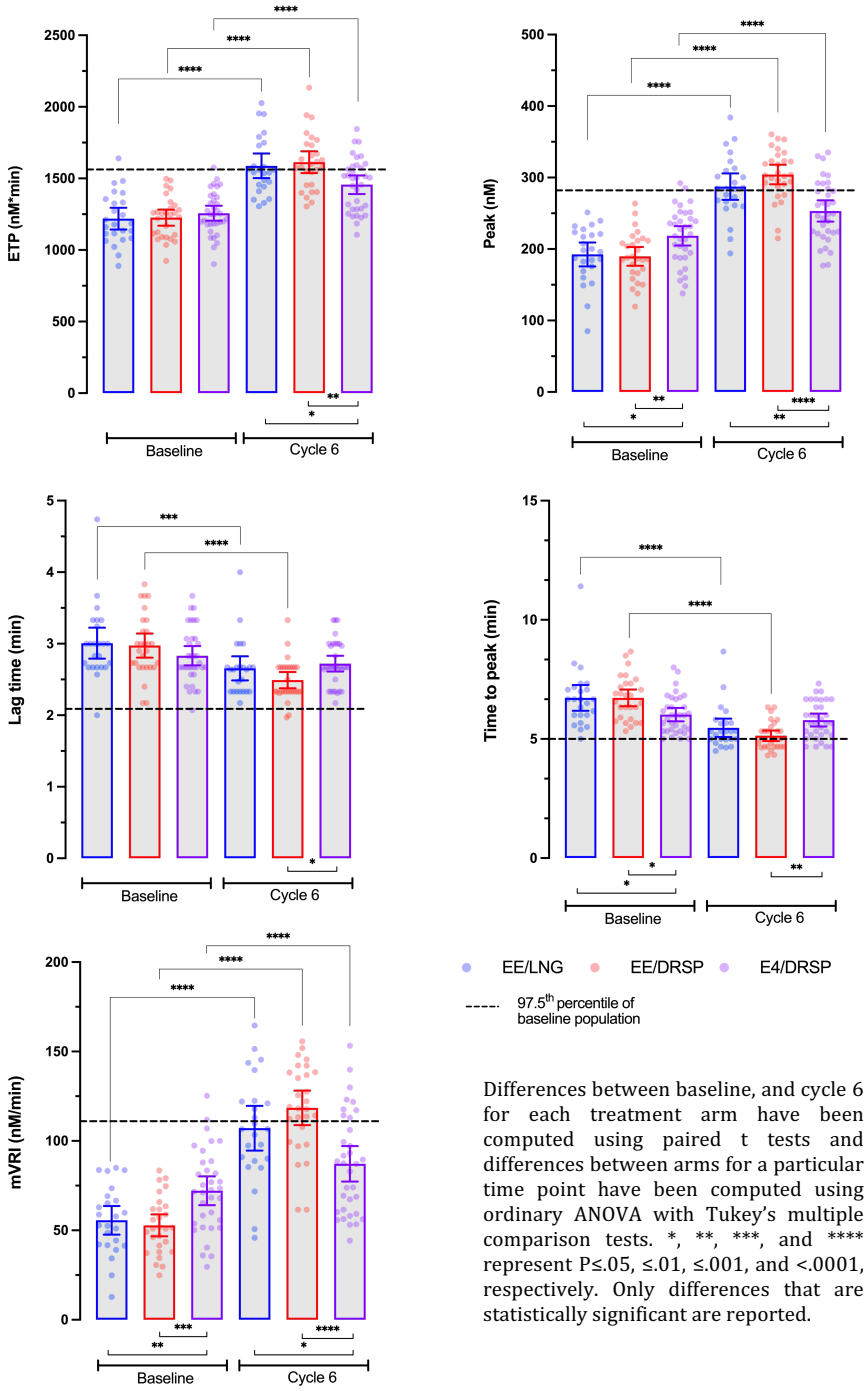
	ETP (nM.min)				Peak height (nM)				Lag time (min)			
	Baseline	**Cycle 6**	***Relative diff. (%)***	***P-value***	**Baseline**	**Cycle 6**	***Relative diff. (%)***	***P-value***	**Baseline**	**Cycle 6**	***Relative diff. (%)***	***P-value***
EE/LNG	1219 ±180	1588 ±204	*32.0 ±20.5*	***<.0001***	192.5 ±39.6	287.3 ±43.7	*53.6 ±29.4*	***<.0001***	3.01 ±0.51	2.65(5) ±0.40	*-10.5 ±12.9(5)*	***.0005***
EE/DRSP	1226 ±146	1613 ±197	*32.8 ±19.0*	***<.0001***	189.7 ±33.8	304.3 ±35.6	*65.5 ±38.4*	***<.0001***	2.97 ±0.43	2.49 ±0.29	*-15.2(5) ±10.4*	***<.0001***
E4/DRSP	1257 ±150	1456 ±186	*16.8 ±15.9*	***<.0001***	218.5 ±39.0	253.2 ±42.6	*17.3 ±16.5*	***<.0001***	2.83 ±0.39	2.72 ±0.32	*-2.4(5) ±15.8*	*.1632*
P-value†	.6167	**.0042**	***.0010***	/	**.0054**	**<.0001**	***<.0001***	/	.2636	**.0275**	***.0013***	/
	Time to peak (min)				**mVRI (nM/min**							
	Baseline	**Baseline**	***Relative diff. (%).***	***P-value***	**Baseline**	**Cycle 6**	***Relative diff. (%)***	***P-value***				
EE/LNG	6.72 ±1.28	5.47 ±0.91	*-18.0 ±8.5*	***<.0001***	55.6 ±19.0	107.1 ±29.6	*105.0 ±56.4*	***<.0001***				
EE/DRSP	6.72 ±0.91	5.13 ±0.58	*-23.0 ±8.1*	***<.0001***	52.8 ±15.8	118.5 ±24.9	*143.6 ±98.8*	***<.0001***				
E4/DRSP	6.02 ±0.80	5.79 ±0.78	*-3.2 ±12.2*	*.0747*	72.2 ±23.0	87.2 ±28.5	*24.3 ±27.9*	***<.0001***				
P-value†	**.0078**	**.0046**	***<.0001***	/	**.0004**	**.0001**	***<.0001***	/				

† P-value had been estimated by using an ordinary one-way ANOVA. A Tukey's multiple comparisons test, with a single pooled variance has been run to assess the difference between the groups. P-value <.05 are considered statistically significant.

Differences at baseline are observed depending on treatment arm: E4/drospirenone arm has statistically higher peak (mean difference versus EE/levonorgestrel: 26.0 nM [95%CI: 2.1-49.9(5) nM] and versus EE/drospirenone: 28.8(5) nM [95%CI: 5.9(5)-51.7(5) nM]), higher mVRI (mean difference versus EE/levonorgestrel: 16.5 nM/min [95%CI: 3.9-29.1 nM/min] and versus EE/drospirenone: 19.4 nM/min [95%CI: 7.3-31.4 nM]) and a shorter time to peak (mean difference versus EE/levonorgestrel: 0.70 min [95%CI: 0.07-1.33 min] and versus EE/drospirenone: 0.70 min [95%CI: 0.1-1.30 min]). After 6 cycles of treatment, no statistically significant difference is observed between EE/levonorgestrel and EE/drospirenone groups (P>.05; **Figure III.2.3**). On the other hand, the ETP, the Peak and the mVRI are significantly lower in the E4/drospirenone group compared to EE/levonorgestrel and EE/drospirenone while the Lag Time and the Time-to-peak are significantly higher in the E4/drospirenone group compared to EE/drospirenone, but not compared to EE/levonorgestrel. In addition, mean values of all TGA parameters are within the reference intervals (2.5^{th}-97.5^{th} percentile of the entire baseline cohort) for E4/drospirenone while mean ETP and Peak are out of ranges for both EE-containing products and the mean mVRI is also outside the reference range for EE/drospirenone. As shown in **Table III.2.3** depending on the TGA parameter, up to 32% of values are outside the reference ranges for E4/drospirenone while they reach 79% for EE/drospirenone and 58% for EE/levonorgestrel.

Table III.2.3 | Reference intervals ([2.5^{th} – 97.5^{th} percentile] of the entire baseline cohort) of thrombin generation parameters and out of range results after 6 cycles of treatment.

	ETP (nM.min)	**Peak Height (nM)**	**Lag time (min)**	**Time to peak(min)**	**mVRI (nM/min)**
Reference ranges	906–1562	119.6–282.0	2.09–3.80	5.00–8.64	24.8–111.0
EE/LNG (n=25)	10 (42%)	14 (58%)	0 (0%)	6 (25%)	10 (42%)
EE/DRSP (n=28)	18 (64%)	22 (79%)	2 (7%)	12 (43%)	19 (68%)
E4/DRSP (n=34)	11 (32%)	9 (26%)	0 (0%)	5 (15%)	9 (26%)

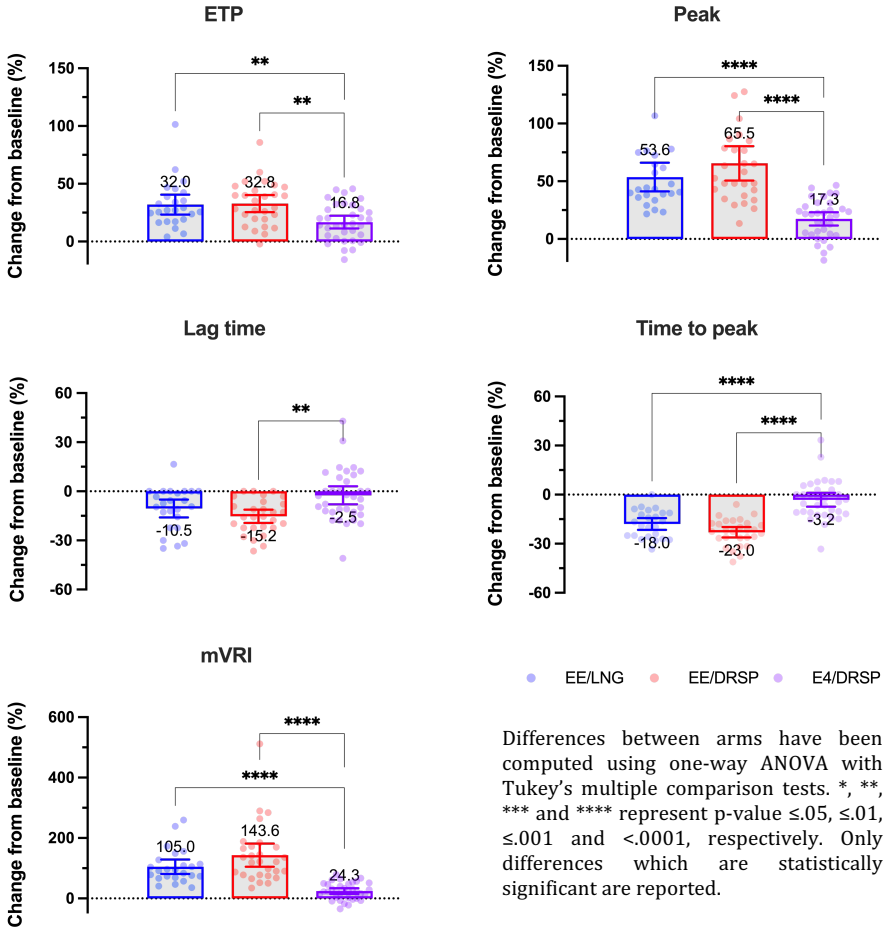


Differences between baseline, and cycle 6 for each treatment arm have been computed using paired t tests and differences between arms for a particular time point have been computed using ordinary ANOVA with Tukey's multiple comparison tests. *, **, ***, and **** represent P≤.05, ≤.01, ≤.001, and <.0001, respectively. Only differences that are statistically significant are reported.

Figure III.2.3 | Graphical representation of all thrombin generation parameters at baseline and after 6 cycles of treatment for the different COC associations.

Change from baseline

Compared to the baseline, the ETP, the Peak and the mVRI are statistically different in all study arms after 6 cycles of treatment. The Lag time and the Time to peak are only statistically reduced in EE/levonorgestrel and EE/drospirenone arms (**Figure III.2.3**). These temporal parameters are not influenced by the intake of E4/drospirenone. These data and the relative changes from the baseline (%) for each TGA parameters are summarized in **Table III.2.2** and **Figure III.2.4.** All TGA parameters are statistically less impacted by E4/drospirenone than EE/levonorgestrel or EE/drospirenone. There is no statistical difference between EE/levonorgestrel and EE/drospirenone.



Differences between arms have been computed using one-way ANOVA with Tukey's multiple comparison tests. *, **, *** and **** represent p-value ≤.05, ≤.01, ≤.001 and <.0001, respectively. Only differences which are statistically significant are reported.

Figure III.2.4 | Change from the baseline (%) of all thrombin generation parameters after 6 cycles of treatment for the different COC associations.

DISCUSSION

Haemostasis is a finely balanced physiological process and even if CHC-induced changes of coagulation factors often remain within the normal range of the population, the supra-additive effect tends to increase the total thrombogenicity. Haemostatic changes induced by CHCs involve levels of fibrinogen, prothrombin, FVII, FVIII, FIX, FX, FXII, AT, protein C, protein S, and TFPI (Douxfils, Morimont, & Bouvy, 2020). Over the past 30 years, many studies have been conducted to assess the impact of the different CHCs on the coagulation system (Douxfils, Morimont, & Bouvy, 2020) and overall, these have revealed that the effect on haemostasis depends on the type of oestrogen, their dose, and their association with progestin (de Bastos et al., 2014; Jick et al., 1995; Kemmeren et al., 2001; Morimont, Haguet, et al., 2021; Norris et al., 1996; World Health Organization Collaborative Study of Cardiovascular Disease and Steroid Hormone Contraception, 1995).

In this study, thrombin generation was assessed in a cohort of women treated for 6 cycles with either E4 in association with drospirenone, EE in association with levonorgestrel or with drospirenone. The global representation of the impact of these therapies on thrombin generation is illustrated by mean thrombograms on **Figure III.2.2.** This simple visual analysis demonstrates the distinct impacts of these therapies on the global coagulation process.

Namely, the mean [±95%CI of the mean] thrombogram of women on E4/drospirenone during 6 cycles does not exceed the reference baseline interval contrary to EE/levonorgestrel and EE/drospirenone groups. This visual evaluation is confirmed by the quantitative analyses performed on the different TGA parameters, i.e., Lag time, Time to peak, Peak, ETP and mVRI (**Table III.2.2** and **Table III.2.3**). After 6 cycles, all TGA parameters were statistically less impacted in the E4/drospirenone group compared to EE/levonorgestrel and EE/drospirenone. Importantly, the changes from the baseline were also always statistically smaller in the E4/drospirenone group whatever the TGA parameter considered. On the other hand, no statistical difference was observed between EE/levonorgestrel and EE/drospirenone although some parameters like the Lag time, the mVRI and to a lesser extent the Time to peak could be less impacted in the EE/levonorgestrel group compared to the EE/drospirenone group but without reaching the significance level.

Thrombograms, however, revealed differences at baseline depending on treatment arm. Indeed, the mean thrombogram at the baseline in the E4/drospirenone group was shown to be higher for the Peak, the Time to peak and the mVRI parameters (**Table III.2.2** and **Figure III.2.3).** This observation is nevertheless mere coincidence as participants were first stratified by previous hormonal contraceptive use and by age, and then the assignment to one of the 3 treatment arms (i.e., E4/drospirenone,

EE/levonorgestrel or EE/drospirenone) was performed using a computerized random allocation sequence. We searched for possible disproportion in the repartition of previous CHC users among the 3 treatment arms. No significant difference was observed between former users and nonusers among each treatment arm, for all TGA parameters (P>.05, unpaired t-tests) except for mVRI in the EE/drospirenone group (P=.046). On the other hand, compared to EE/levonorgestrel and EE/drospirenone, the mild observed hypercoagulable state at the baseline, along with the smaller impact on thrombin generation after 6 months of treatment, reinforce the conclusion that E4 in association with drospirenone induces fewer procoagulant changes than EE/levonorgestrel or EE/drospirenone. This should be perceived as an additional advantage of E4/drospirenone over EE/levonorgestrel and EE/drospirenone since the absolute thrombogram after 6 cycles is lower than the ones of EE/levonorgestrel and EE/drospirenone even in a population showing a more procoagulant state at baseline.

Added to the results on the APCR[2], these data mean that the global effect of an association of 15mg E4 with 3mg drospirenone has less impact on the entire coagulation than EE 30µg with levonorgestrel 150µg and EE 20µg with drospirenone 3mg. The mean thrombogram of the E4/drospirenone group, along with all TGA parameters, standing within the reference ranges clearly delineates the neutral profile of this association on thrombin generation. Mean changes in ETP, Peak height, Lag time and Time to peak were below 20% while mVRI (a very sensitive TGA parameter) was only impacted by 24% whereas it is impacted by 105% and 144% in the EE/levonorgestrel and the EE/drospirenone groups (**Table III.2.2**). Moreover, the different thrombograms along with TGA parameters between E4/drospirenone and EE/drospirenone strongly indicates that the lower impact of the new approved CHC, i.e., 15mg E4/3mg drospirenone is attributable to the difference in its oestrogenic content and is not related to drospirenone. Besides, the use of 4mg drospirenone alone revealed to be associated with a low impact on haemostasis parameters (Chiara Del Savio et al., 2020; Palacios et al., 2019).

The fact that TGA parameters were not significantly influenced by the intake of E4/drospirenone over the 6-month course of this study is very reassuring. The LETS study (Leiden Thrombophilia Study) revealed that patients with ETP above the 90th percentile on the control population were more prone to have a recurrent thrombotic event, suggesting a correlation between the risk of recurrent VTE and high ETP (van Hylckama Vlieg et al., 2007). In the combined LITE (Longitudinal

[2] These results demonstrated that the E4/drospirenone combination has statistically less impact on the nAPCsr than EE/levonorgestrel and EE/drospirenone after 3 or 6 months of treatment.

Investigation of Thromboembolism Etiology) data, participants with Peak height values above the median were at 74% greater risk of VTE, as compared to those in the lowest quartile (Lutsey et al., 2009) suggesting that compounds with the highest impact on thrombin generation are also those who are more prone to be associated with thromboembolic events. This is also supported by our recent prediction models using the nAPCsr as surrogate marker for the risk of VTE (**Part II**). There is much other evidence that higher thrombin generation profiles, reflected by higher ETP, Peak height or mVRI and reduced Lag time or Time to peak, are associated with an increased risk of occurrence or recurrence of thrombosis (Eichinger et al., 2008; Hron et al., 2006; Negrier et al., 2017; Tripodi et al., 2008; Tripodi et al., 2007).

Although a comparative phase IV study (i.e., post approval study) will be necessary to definitively prove the lower VTE risk profile of 15mg E4/3mg drospirenone, the fact that no VTE event was observed in the US phase III study evaluating E4/drospirenone in 1864 women is already reassuring. Also 23% of the study participants had a BMI higher than 30.0 kg/m^2(Creinin et al., 2021) a well-known risk factor for VTE (Abdollahi et al., 2003), suggesting a low incidence rate using this formulation. Previous studies of the same magnitude in similar populations using low risk CHCs (e.g., EE 10µg/norethindrone acetate 1mg, a vaginal ring delivering EE 13µg and segesterone acetate 150µg per day or EE 30µg/levonorgestrel 120µg) have reported higher absolute numbers of VTE events. Three thrombotic events were reported among 1683 (0.2%) US women using EE 10µg/norethindrone acetate 1mg, of whom 18% were obese (Archer et al., 2013). Four VTE occurred among 1188 (0.3%) US women using a vaginal ring delivering EE 13µg and segesterone acetate 150µg per day (Gemzell-Danielsson et al., 2019) and 4 VTE occurred among 2031 (0.2%) US women, of whom 35% were obese, using a new contraceptive patch with dosing equivalent to an EE 30µg/levonorgestrel 120µg oral contraceptive pill (Nelson et al., 2021). Thus, even with low dose EE-containing CHCs, the thrombotic risk is still elevated substantially and higher than the one observed during the clinical development of E4/drospirenone. This may be in part explained by the unique mode of action of E4 which acts differently on the liver, potentially reducing the risk of VTE.

However, in the real world (i.e., outside the setting of the clinical study in which exclusion criteria permit selecting a low-risk population) the inclusion of women with an unknown coagulopathy at the time of contraception initiation is unavoidable due to the current screening strategies. As previously described (Morimont, Haguet, et al., 2021), the presence of a genetic mutation (i.e., FVL, prothrombin G20210A mutations, protein C, protein S and antithrombin deficiencies) with CHCs leads to a synergistic and amplificative (rather than an additive) prothrombotic effect. Although contraindicated, cases of exposure in such population will occur and may

inform on the amplificative effect of these prothrombotic conditions in presence of E4/drospirenone. In a perspective, this could allow the computation of a synergy index for E4/drospirenone, similar to what was done by *Hugon-Rodin et al.*(Hugon-Rodin et al., 2018) and *Khialani et al.* (Khialani et al., 2020) with other CHC preparations. Finally, this will permit us to appreciate the contribution of E4/drospirenone on the global prothrombotic profile of a woman.

CONCLUSION

In conclusion, an association of 15mg E4 with 3mg drospirenone does not have an impact on thrombin generation compared to EE-containing products which, either associated with levonorgestrel or drospirenone, are able to increase the production of procoagulant factors and decrease the production of anticoagulant ones, shifting the patient to a prothrombotic state. Ethinylestradiol-containing products thus generate prothrombotic environments contrary to E4 which demonstrates a neutral profile on haemostasis. Although this must be confirmed by data obtained from a post-approval study, a previous experience with oestradiol in association with nomegestrol acetate has permitted to validate the concept that these surrogate biomarkers may reflect the VTE profile of a specific CHC compared to a reference association (Gaussem et al., 2011; Reed et al., 2021). This further suggests that E4/drospirenone is less likely to be associated with VTE risk compared to EE-containing products.

PART IV: PERSPECTIVES AND CONCLUSION

INTEREST IN OTHER HORMONE-INDUCED COAGULOPATHIES

HORMONE THERAPY IN MENOPAUSAL WOMEN

The use of menopausal replacement therapy (MRT) remains a matter of concern regarding the risk of VTE. Compared to nonusers, oral preparations containing either conjugated equine oestrogen (CEE) or oestradiol (E2) are associated with an increased risk of VTE. In non-hysterectomized women, combinations of CEE or E2 with medroxyprogesterone acetate or norethisterone further increase the risk of VTE, underlying the impact of the associated progestin on the prothrombotic risk. (Manyonda et al., 2020; Vinogradova et al., 2019).

As with combined oral contraceptives (COCs), MRT induce changes in plasma levels of almost all proteins involved in coagulation and fibrinolysis (Blondon et al., 2014; Canonico, 2015; Harrington et al., 2017; Smith et al., 2014). Increases in FII, FVIII or FX levels and/or decreases in protein S and TFPI levels induce acquired activated protein C resistance (APCR) (Brenner, 2004; de Visser et al., 2005; Douxfils, Morimont, & Bouvy, 2020; Morimont, Haguet, et al., 2021) and this phenotype, both in COC- and MRT-users, has been linked to an increased risk of VTE (Canonico et al., 2010; de Visser et al., 2005; Douxfils, Morimont, & Bouvy, 2020; Eilertsen et al., 2019; Morimont, Dogné, et al., 2020; Morimont, Haguet, et al., 2021).

In 2021, estetrol (E4) in association with drospirenone was commercialized as a new COC. The favourable profile of E4-containing product on haemostasis has been discussed in **Part III**. Nevertheless, this new active substance also showed promising results to relieve postmenopausal symptoms such as vasomotor symptoms (VMS) (Abot et al., 2014; Arnal et al., 2017; Foidart et al., 2019; Gerard et al., 2022) and genitourinary syndrome of menopause (GSM) (Gaspard et al., 2022). Last December 22nd, Gedeon Richter Plc. and Mithra Pharmaceuticals announced that they had signed a Binding Term Sheet for the commercialization of Donesta®, an orally-administered E4-based MRT product (*Richer and Mithra sign binding Heads of Terms for commercialization of Donesta*).

Apart from its beneficial effect for relieving postmenopausal symptoms, the E4Relief Phase II trial confirmed that E4 had a low impact on haemostasis (Douxfils, Gaspard, et al., 2022). The endogenous thrombin potential (ETP)-based APCR assay revealed a relative increase of the normalized activated protein C sensitivity ratio (nAPCsr) of 41.9% with the highest dose of E4 (i.e., 15mg), after 12 weeks of treatment. This increase was similar to the increase observed after 3 cycles (corresponding approximately to 12 weeks of treatment) in women treated with E4 15mg/drospirenone 3mg (i.e., relative increase of 39.5%) (**Figure IV.1**). Compared to ethinylestradiol (EE)-containing products used in contraception for which the nAPCsr

was increased by 165% (EE/levonorgestrel) and 229% (EE/drospirenone) at cycle 3, E4 either administered alone in postmenopausal women or in combination with drospirenone in contraception seems to have a neutral profile on this global coagulation marker (Douxfils, Klipping, et al., 2020).

As shown in **Figure IV.1**, the impact of other MRT on the nAPCsr has been previously evaluated, although it was not with the new sensitive method developed by QUALIblood s.a. (Douxfils, Morimont, Delvigne, et al., 2020) *Post et al.* reported a 28% relative increase with transdermal E2 at a dose of 50µg (Post, Christella, et al., 2003), and increases above 100% with different oral preparations containing 17β-E2, alone or in association with a progestin (Post, Christella, et al., 2003; Post et al., 2002; Post, van der Mooren, et al., 2003). These data support the hypothesis that oral E4, with its different pharmacological profile compared to oral CEE or oral E2, has a lower impact on the liver and on haemostasis.

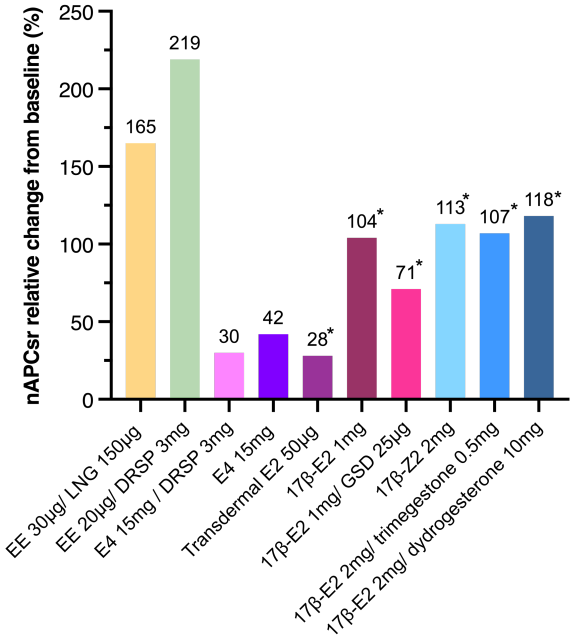


Figure IV.1 | nAPCsr relative change from baseline of COCs and MRTs.
*(Post, Christella, et al., 2003; Post et al., 2002; Post, van der Mooren, et al., 2003)

As discussed in **Part III**, the ETP-based APCR assay also permits the extraction of thrombin generation data (using thrombograms generated under the negative-APC condition), allowing the assessment of the coagulation process in its entirety. As TGA has been shown to be sensitive to the synergistic haemostatic alterations induced by MRT (Hemker et al., 2003), we studied the impact of multiple doses of E4 (issued from the E2Relief phase II trial) on thrombin generation.

Population (i.e., postmenopausal women) included in this trial being different compared to the one described in **Part III** (i.e., women of childbearing age); new reference ranges (2.5th-97.5th percentile) were established based on baseline thrombograms of this cohort of postmenopausal women (n=131) (**Figure IV.2**).

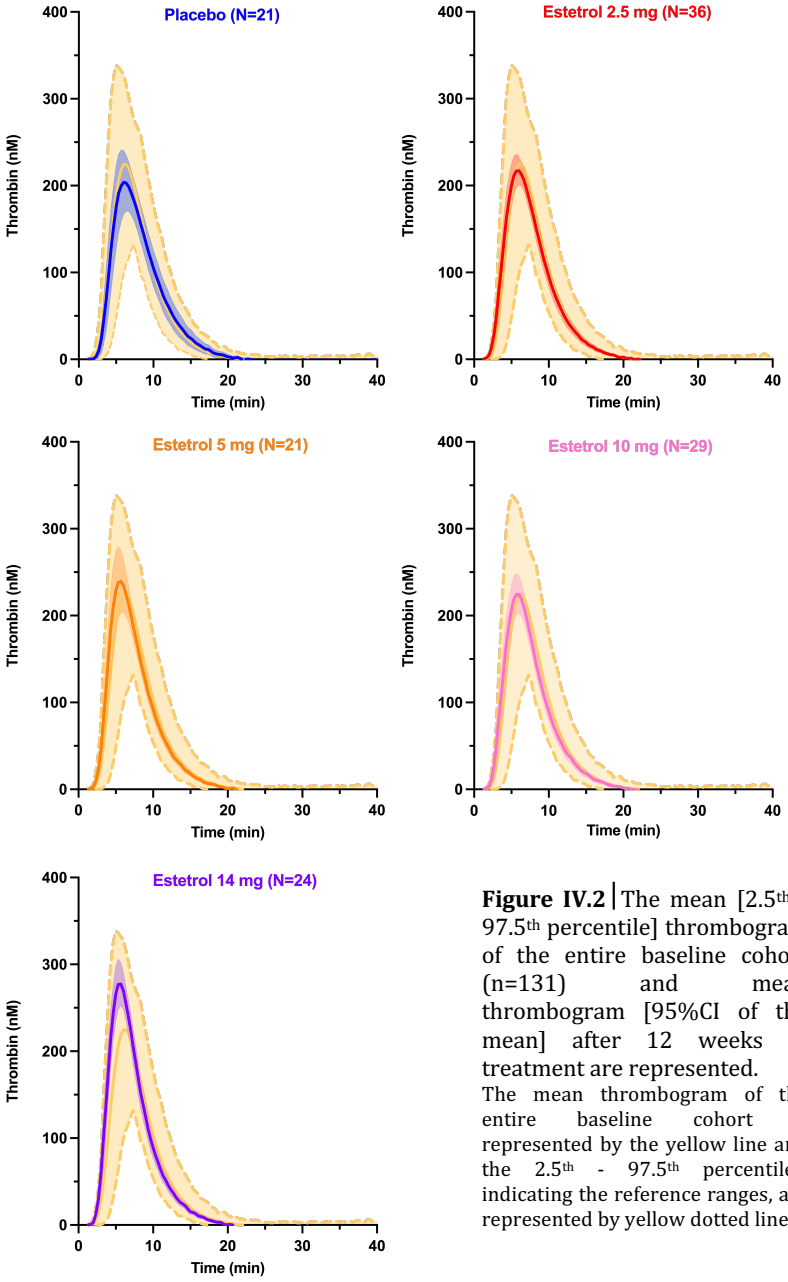


Figure IV.2 | The mean [2.5th - 97.5th percentile] thrombogram of the entire baseline cohort (n=131) and mean thrombogram [95%CI of the mean] after 12 weeks of treatment are represented.
The mean thrombogram of the entire baseline cohort is represented by the yellow line and the 2.5th - 97.5th percentiles, indicating the reference ranges, are represented by yellow dotted lines.

After 12 weeks of treatment with E4 (2.5, 5, 10 and 15mg) or placebo, mean thrombograms remained within the reference ranges, supporting the claim that E4 has no clinically relevant impact on haemostasis in this population (**Figure IV.2**).

The evolution of the different TGA parameters from baseline to week 12 are shown in **Figure IV.3**. At the level of the individual, the impact of E4 on thrombin generation is limited since few subjects have abnormal TGA parameters after 12 weeks of treatment (n=13; ≥3 TGA parameters outside reference ranges). Also, for 3 of these patients, at least 1 TGA parameter was already outside the reference range at baseline.

Changes from baseline were statistically significant compared to placebo for all TGA parameters with the highest dosage of E4 (**Figure IV.5**). However, the high thrombin generation curves observed in the placebo group at baseline undoubtedly influenced the change from baseline in this group and therefore influence the difference between the E4 groups and the placebo when comparing the changes from baseline. This observation is confirmed by the fact that when comparing only the time point after 12 weeks of treatment (**Figure IV.4**), none of the E4 doses showed statistically different ETP, Peak, Time to peak, Lag time or mVRI results compared to the placebo group.

This study on thrombin generation confirms the current evidence of the weak impact of E4 on haemostasis in postmenopausal women. After having demonstrated that E4 only minimally influences nAPCsr, these results reveal that its impact on thrombin generation, a global coagulation test sensitive to the changes induced by oestrogenic compounds, is negligible and not clinically relevant. This is in line with the data obtained with E4 in contraception (and discussed in **Part III**) which suggested a safer haemostatic profile compared to EE. When comparing these results with the literature, it appears that oral E4 has probably a similar impact on thrombin generation than transdermal E2 (Scarabin et al., 2011). Obviously, a direct comparison would be required to confirm this observation but the current knowledge about its pharmacological profile provide rational to this statement. Further studies are needed to confirm these results, but accumulating evidence is reinforcing the message that E4 has a neutral profile on haemostasis. Results from the phase III program will provide important additional information on the safety profile of E4 in menopause.

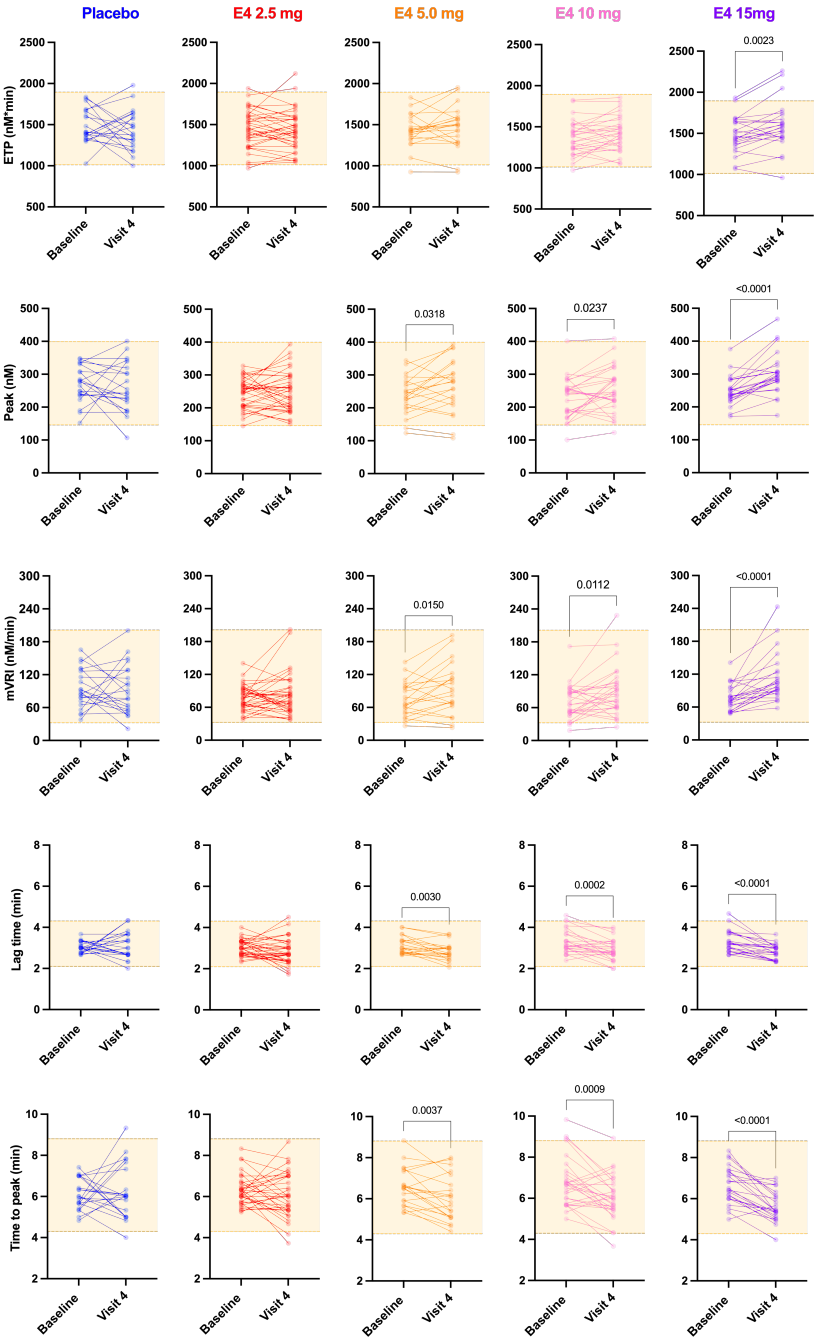


Figure IV.3 | TGA parameters at baseline and after 12 weeks of treatment (placebo or E4 2.5 mg, 5mg, 10mg and 15 mg) for all individuals.

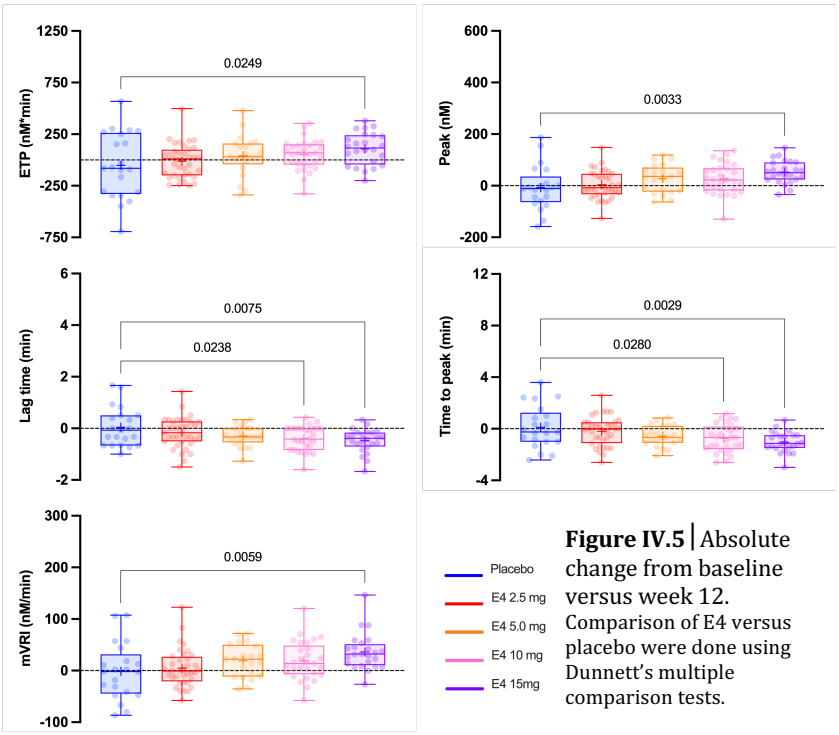


Figure IV.5 | Absolute change from baseline versus week 12. Comparison of E4 versus placebo were done using Dunnett's multiple comparison tests.

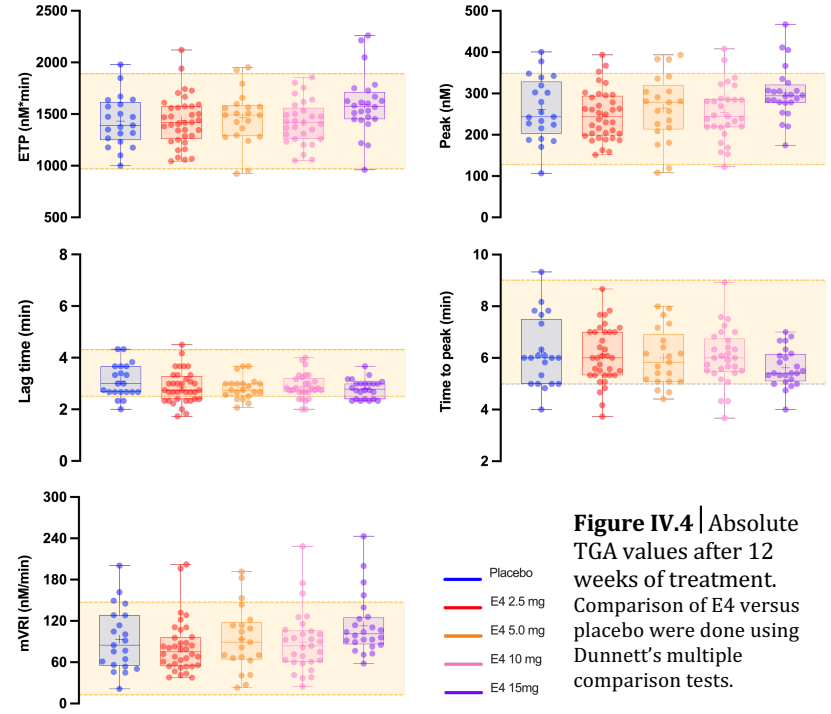


Figure IV.4 | Absolute TGA values after 12 weeks of treatment. Comparison of E4 versus placebo were done using Dunnett's multiple comparison tests.

TAMOXIFEN IN OESTROGEN DEPENDENT BREAST CANCER PATIENTS

Breast cancer is the most prevalent cancer in women worldwide, and oestrogen receptor (ER) positive (ER+) breast cancer represents approximately 75% of all breast cancer diagnosed (Institute, 2019; Patel et al., 2018). Overall, women diagnosed with breast cancer have a 3- to 4-fold increased risk of VTE as compared to healthy women of an equivalent age (Cronin-Fenton et al., 2010; Walker et al., 2013). Previous studies have identified several risk factors of VTE in breast cancer patients, including the use of tamoxifen (Walker et al., 2016). Despite being generally well tolerated, tamoxifen use is associated with a higher incidence of VTE compared to nonusers or users of other therapy, such as aromatase inhibitors (Lin et al., 2018; Onitilo et al., 2012). During the first 3 months of tamoxifen therapy, a 5-fold increased risk of VTE has been observed (Walker et al., 2016) and at five years, the risk is reportedly 2 to 3-fold compared to nonusers (Cuzick et al., 2007; Duggan et al., 2003; Mandala et al., 2012; Veronesi et al., 2007). Additionally, the risk of VTE was found to be approximately 1.4-fold higher than in patients taken aromatase inhibitors (Xu et al., 2019).

The recognized impact of tamoxifen on haemostatic parameters involves an increase in coagulation FVIII levels as well as decreases in antithrombin, TFPI and protein C levels (Cosman et al., 2005; Cushman et al., 2003; Erman et al., 2004; Mannucci et al., 1996). There are conflicting results regarding fluctuations in fibrinogen and protein S levels as well as APCR (Blondon et al., 2022; Cushman et al., 2003; Ruhl et al., 2014). The latter could be attributed to the lack of a standardized methodology as it was the case with COCs and extensively discussed in **Part I**. Now that the ETP-based APCR assay is validated, we assessed the effect of tamoxifen on APCR and on TGA parameters (in absence and presence of exogenous APC) in patients receiving tamoxifen as adjuvant treatment of ER+ breast cancer following successful tumour resection.

Overall, 25 patients in remission from ER+ breast cancer using tamoxifen as adjuvant therapy for at least 4 weeks without interruption were included. Among the 25 patients, 2 were known to have a heterozygous FV Leiden (FVL) mutation.

To assess the impact on nAPCsr and TGA parameters, an age- and BMI-matched control group was used. This control group corresponded to the cohort of postmenopausal women (n=131), as described above.

As shown in **Figure IV.6,** thrombograms revealed a hypercoagulability shift as evidenced by increased thrombin generation in patients treated with tamoxifen as compared to the control group.

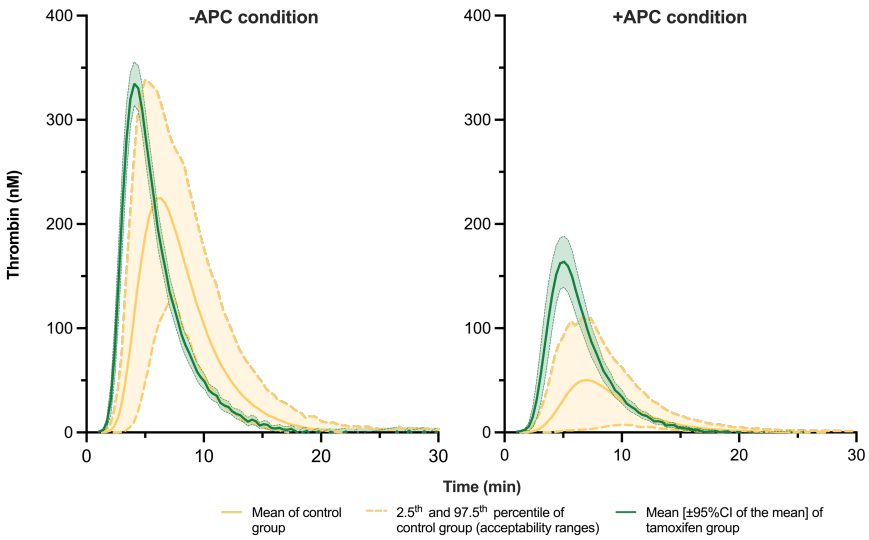


Figure IV.6 | Thrombograms in absence and presence of exogenous APC in tamoxifen users versus the reference population

Mean thrombograms of the control group (n=131) is represented by the continuous yellow line, while the 2.5th – 97.5th percentiles are represented by the dotted yellow line. The green line represents the mean ± 95%CI thrombogram of patients taking tamoxifen (n=23).

This was confirmed by the quantitative analysis of the TGA parameters, presented in **Figure IV.7.** The higher peak height observed in the tamoxifen group is consistent with the results of previous studies, which showed an association of increased peak height with higher risk of a first deep venous thrombosis, in patients with cancer (Ay et al., 2011; Hron et al., 2006; Lutsey et al., 2009; Pabinger et al., 2013; van Hylckama Vlieg et al., 2007) and those without (Tans et al., 2003). The shorter lag time observed in the tamoxifen group could also be related to decreased TFPI levels, as low plasma circulating levels of this anticoagulant protein potentiate the initiation phase of the coagulation, therefore contributing to a prothrombotic profile (Dahm et al., 2003; de Visser et al., 2005; Dielis et al., 2007).

In presence of exogenous APC, the mean thrombogram was within the upper part of the reference range **(Figure IV.6)** and the individual TGA parameter analysis revealed a mean ETP above the upper limit (**Figure IV.7**). This resulted in a significantly higher nAPCsr value for the tamoxifen group (mean ±SD, 3.18 ±0.91) compared to the control group (mean ±SD, 2.19 ±0.92).

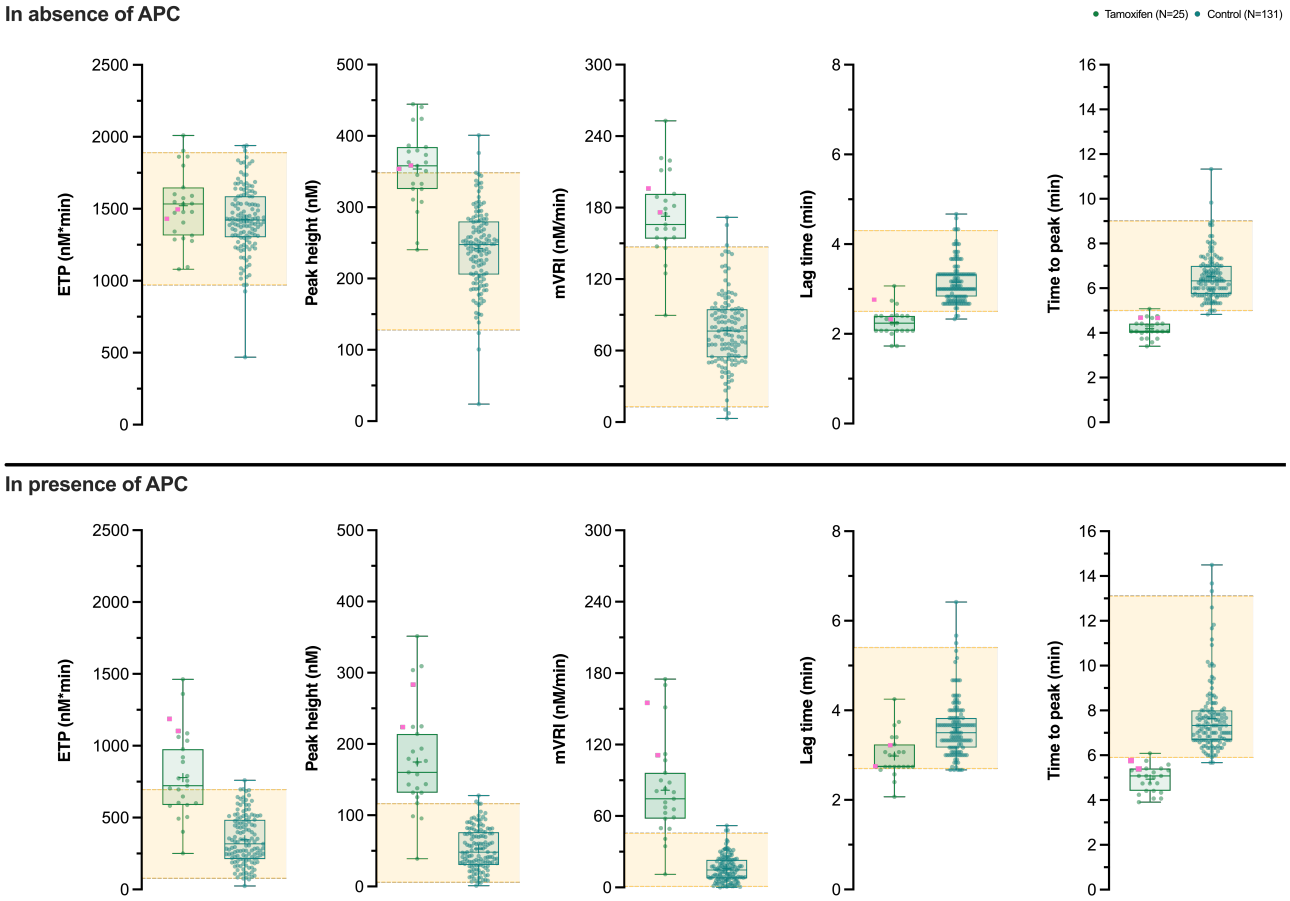


Figure IV.7 | TGA parameters in absence and presence of APC in tamoxifen users (n=25) and healthy individuals (n=131). The median and the 25th - 75th percentiles are symbolized by boxes; whiskers represent minimum and maximum values. The mean value is represented by a cross. Pink squares represent FVL carriers. Yellow dotted lines represent reference ranges (2.5th-97.5th percentile of control group)

This acquired APCR observed in patients taking tamoxifen could be explained, at least in part, by decreases in TFPI. The anticoagulant activity of TFPI lies in its ability to inhibit factor Xa and subsequently TF and factor VIIa by forming an inactive FXa-TFPI-TF-FVIIa quaternary complex. When TFPI levels are reduced, there is an increase in FXa levels, and FXa has shown to protect FVa from inactivation by APC. Recent data also reports that protein arginine deiminase type IV (PAD-4), a protein involved in neutrophil extracellular traps (NETs) (Morimont, Dechamps, et al., 2022) may lead to TFPI modification at arginine 107, which results in loss of anti-factor Xa activity (Thomassen et al., 2023). As NETosis has been reported in women with breast cancer (Snoderly et al., 2019), one cannot exclude that even patients in remission may be more prone to generate NETs, further contributing to this procoagulant profile and a reduced sensitivity to APC (de Visser et al., 2005; Didembourg et al., 2021). Another factor known to influence the ETP-based APCR assay is free protein S levels (de Visser et al., 2005). As also described in **Part I.4**, this protein normally forms a complex with APC to efficiently inactivate FVa and FVIIIa, and if protein S levels are decreased, the proteolytic cleavage of FVa by APC is no longer effective, resulting in delayed FVa inactivation and increased thrombin formation (de Visser et al., 2005). Nevertheless, the impact of tamoxifen on protein S levels is controversial, and this proposed explanation thus remains hypothetical.

In this study, two patients were heterozygous for the FVL mutation, and they expressed nAPCsr values of 4.40 and 4.89. These high nAPCsr values, standing above the 75th percentile of the entire cohort study, were consistent with our assumptions that tamoxifen in the setting of FVL mutation may have a synergistic effect as observed in women using COCs (Hugon-Rodin et al., 2018; Khialani et al., 2020) and previously discussed. Indeed, in **Part I.4**, we showed that higher nAPCsr values were obtained in women with heterozygous FVL mutation taking COCs as compared with COC use or FVL mutation alone (Morimont, Donis, et al., 2022). A similar association might therefore exist between FVL mutation and tamoxifen use through its oestrogenic agonist activity, which is thought to be responsible for the increased risk of VTE (Eroglu et al., 2011; Eroglu et al., 2003). In addition, a case-control study by Garber *et al.* demonstrated that women taking adjuvant tamoxifen for early-stage breast cancer had an increased risk of VTE if they carried the FVL mutation (OR of 4.73) (Garber et al., 2010).

This study is however limited by its small sample size, as well as the lack of a real time matched control. In the future, a longitudinal cohort study including patients before the initiation of tamoxifen therapy would be required to confirm our exploratory findings. This would allow for the calculation of the baseline risk of VTE in these patients, and subsequent testing at several time points during treatment

would provide a detailed assessment of the impact of tamoxifen on haemostasis over time.

In conclusion, this study demonstrated that tamoxifen therapy induced an acquired APCR, suggesting that the aetiology of tamoxifen induced-VTE is likely like that of COCs. Further clinical research remains essential to consider the nAPCsr as a universal scale to determine individual VTE risk when being considered for hormonal therapies characterized by an oestrogenic activity, including tamoxifen and possibly other selective oestrogen receptor modulators.

PREGNANT WOMEN

Pregnancy, delivery, and postpartum are associated with many haemostasis complications as well as significant morbidity or mortality to both mother and foetus (Pomp et al., 2008). VTE occurs in approximately one in 100 pregnancies, and pulmonary embolism is a leading cause of maternal death (Kujovich, 2004). The risk of VTE is 5-fold higher during pregnancy and up to a 20- to 60-fold in the postpartum period (i.e., during the first 6 weeks after delivery) compared to non-pregnant women (Heit et al., 2005; Jackson et al., 2011; Pomp et al., 2008).

During normal pregnancy, the haemostatic balance changes to a hypercoagulable state to limit bleeding complications at delivery. This shift to a prothrombotic tendency is explained by increases in numerous coagulation factors (FVII, FVIII, FX, Von Willebrand and fibrinogen levels); decrease of protein S activity and reduced fibrinolytic activity (by increases in PAI-1 and placenta-derived PAI-2 and a decrease in t-PA). Markers of in vivo thrombin generation, i.e., prothrombin fragment 1+2 and thrombin-antithrombin (TAT) complexes, are also increased. (James, 2009; Rosenkranz et al., 2008). An APCR phenotype, using an aPTT-based assay, has also been highlighted in pregnant women compared to healthy non pregnant ones (Mathonnet et al., 1996).

Due to this physiological hypercoagulable state during pregnancy, it is obvious that thrombophilia represents a non-negligible additional risk factor of VTE. The most common hereditary thrombophilia observed in women with pregnancy associated VTE are heterozygosity for FVL and prothrombin (PT) gene mutation and high FVIII levels (>150% of normal activity). At least 50-75% of thrombotic episodes in carriers of FVL or PT G20210A mutations are triggered by additional predisposing factors, with pregnancy as the most common circumstantial risk factor (Kujovich, 2004). Being able to identify these mutations before, or at least right after the 1st appointment to the gynaecologist could improve the follow-up of the pregnancy to reduce the occurrence of thrombotic events by providing closer monitoring.

Most studies investigating haemostasis during pregnancy assess coagulation and fibrinolytic factors levels individually and as already explained in previous sections, this renders the interpretation of the results very challenging. In 2010, Szecsi et al., reported gestational age-specific reference intervals for 18 haemostatic laboratory tests (aPTT, PT, Owren PT, Quick PT, fibrinogen, D-dimers, antithrombin, free and total protein S and protein S activity, FII, FV, FVII, FVIII, FIX, FX, FXI and FXII levels) (Szecsi et al., 2010). A total of 7 blood samples were attempted for each woman, covering the entire pregnancy, delivery, and postpartum period. Reference intervals corresponding to the 2.5th and 97.5th percentile of the cohort, were calculated for each test and for the gestational periods and included only uncomplicated pregnancies. The most affected parameters, i.e., which stood outside the expected ranges (defined in a non-pregnant population) were fibrinogen, D-dimers, protein S activity, FVII, FVIII and FIX levels. This study confirmed that these physiological changes occurring during pregnancy and postpartum are reflected in several coagulation tests, making usual reference intervals not relevant, hindering accurate diagnosis and treatment. On this basis, it would seem interesting to propose a global coagulation test as substitute. Also in 2010, Rosenkranz et al. investigated thrombin generation in normal uncomplicated pregnancy using the thrombin calibrated thrombogram and PPP reagent as triggering reagent (±5pM TF and 4µM PLs). They showed that ETP and peak height parameters were significantly impacted with gestational age. (Rosenkranz et al., 2008) However, the number of patients included in this study was limited, and there was only one time point. A larger study investigating the potential of TGA as a mean to predict alterations of the clotting system during pregnancy would be necessary.

In this context, the University of Namur in collaboration with QUALIblood s.a. and the Centre Hospitalier Régional (CHR) of Huy (Huy, Belgium) started a single centre longitudinal study to assess the baseline and evolution of the thrombotic risk of pregnant women using the ETP-based APCR assay.

The aim of this study is to assess the coagulability status of women during pregnancy and postpartum period using a panel of coagulation testing[3] including the ETP-based APCR assay and to compare results with the standard procedure (which includes aPTT testing, international normalized ratio (INR) determination and fibrinogen levels measurement). In other words, the aim would be to determine whether the ETP-based APCR assay or other more specific coagulation tests could replace routine haemostasis testing to estimate the patient's procoagulable state and the risk of VTE during pregnancy. In addition, with a sufficiently large number of uncomplicated

[3] Additional tests, such as the FibWave, prothrombin fragment 1+2, t-PA, PAI-1, and protein S levels will be performed.

pregnancies, we will be able to propose reference ranges for many haemostatic parameters including nAPCsr, thrombin generation curve and TGA parameters and this, according to gestational age.

Secondary objectives will consist of analysing the clot formation and assess the changes of these haemostasis laboratory markers throughout the entire pregnancy and postpartum.

From a practical point of view, at least 150 pregnant women will be enrolled at the CHR of Huy. Blood specimens will be collected at inclusion, and at every check-up for 15 months (to cover postpartum period) to fit with the standard of care follow-up surveillance, thus avoiding additional interventions. In addition, 150 plasmas from healthy non-pregnant women will be provided by the Namur Biobank-eXchange (NAB-X), the registered biobank from the University of Namur (Namur, Belgium).

This study has received approval from the ethics committee of the CHR Huy, and patients' recruitment should start shortly.

BEYOND HORMONAL THERAPIES

The APC resistance phenotype is not only observed with the use of hormone therapy and this phenomenon has already been described in several pathological conditions.

Antiphospholipid syndrome is characterized by occurrence of thrombosis and/or pregnancy morbidity with the persistent presence of antiphospholipid antibodies (Vandevelde et al., 2022). Antiphospholipid antibodies are autoantibodies that are directed against phospholipid-binding proteins. There are numerous types of antiphospholipid antibodies including anticardiolipin antibodies associated or not with anti β2-glycoprotein I (β2GPI) antibodies, anti-phosphatidylethanolamin, antiphosphatidylserin/prothrombin, or anti-protein S antibodies. Those with *in vitro* anticoagulant properties (e.g., anti-β2GP or prothrombin antibodies), are referred to as lupus anticoagulant (Male et al., 2001; Nojima et al., 2009; Nojima et al., 2005).

The presence of these antibodies is considered pathogenic as they play a substantial role in thrombosis occurrence, as they are not just serological markers of antiphospholipid syndrome. One plausible mechanism hypothesized to explain the antiphospholipid-induced thrombosis is an acquired resistance to APC. (Male et al., 2001) As for the aetiology of APCR, several postulates were raised. Anti-β2GPI could induced a conformational change of β2GPI, increasing its affinity for anionic phospholipids (PLs) on endothelial cell surfaces. This would lead to a competition with components of the APC system, as binding sites on the PLs surface are limited (de Laat et al., 2008). Similar phenomena were also observed with anticardiolipine/β2GPI and antiphosphatidylserin/prothrombin antibodies enhancing the binding of β2GPI and prothrombin to negatively charged PLs (Devreese et al., 2017; Nojima et al., 2005). In other words, theses antibodies-antiphospholipid-binding protein complexes may lead to indirect inhibition of PLs-dependant reactions of the APC pathway, slowing down the inactivaiton of FVa and FVIIIa. Anti-protein S antibodies would act differently by being responsible for a protein S deficiency, with an effect on protein S activity rather than protein S antigen levels (Nojima et al., 2009). These assumptions represents an open door for the nAPCsr as the ETP-parameter, reflecting the total amount of thrombin generated over time might be a better indicator of the thrombotic risk than clotting times, which only reflect the initiation phase of the coagulation (Regnault et al., 2003; Velasco-Rodriguez et al., 2020; Wahl et al., 2009; Zuily et al., 2012).

Another disease well known for its high heterogeneity is cancer. The risk of VTE in cancer patients is approximately 4-fold that of a healthy population and is even higher with haematological malignancy. The underlying aetiology of cancer-induced thrombosis is complex but one of the underlying mechanisms could be attributed to

acquired APCR. This resistance to APC can be explained in different ways depending on the tumour. For example, increases in procoagulant factors levels (and especially factor FVIII) observed in patient with solid tumours seems to contribute to APCR while in patients with haematological malignancies, APCR is more likely to be associated with decreases in protein S and TFPI levels (Negaard et al., 2008). Also, NETosis, a complex immune process, is very likely to be involved in cancer-associated thrombosis. The enzyme peptidyl arginine deiminase 4 (PAD4), which is an important contributor of the NET formation, is highly expressed in many cancers (Demers et al., 2014). It was suggested that PAD4 may lead to TFPI modification at arginine 107, resulting in a loss of anti-factor Xa activity (Thomassen et al., 2023), further contributing to this procoagulant profile and a reduced sensitivity to APC. Through this heterogenous pattern, the ETP-based APCR assay might be relevant. Indeed, with its low specificity but high sensitivity, the test could detect any APCR phenotype regardless of the cause while conventional aPTT-based assay would probably not.

TOWARDS A BIOMARKER QUALIFICATION

In October 2022, the plasma coagulation inhibitors subcommittee of the International Society on Thrombosis and Haemostasis Scientific and Standardization Committee made a communication on FVL independent-APCR evaluation, and the ETP-based APCR assay stood apart (Moore et al., 2022).

The efforts our team has put on for more than 4 years to demonstrate the interest of the nAPCsr as an assessment tool of COC-associated thrombotic risk, have finally paid off. Nevertheless, this test is not yet available in most laboratories because its implementation into routine clinical practice requires it to be recognized as a biomarker for VTE risk assessment.

Its validation as a biomarker by regulatory authorities would clearly simplify its harmonized use across CHC-developers and would represent a major step for its use in precision medicine (Bakker et al., 2022).

As defined by the National Institutes of Health (NIH) Biomarkers Definition Working Group, a biomarker is "*a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic intervention*" (Baker et al., 2020). The nAPCsr clearly fits with this definition and could fall under the term of safety biomarker and reasonably likely surrogate endpoint (Bakker et al., 2022; FDA-NIH Biomarker Working Group, 2020).

According to the BEST (Biomarkers, EndpointS and other Tools) definition (FDA-NIH Biomarker Working Group, 2020), a safety biomarker is measured before or after exposure to a medical intervention or environmental agent to indicate the likelihood

of an adverse event occurrence. The periodic monitoring of safety biomarkers is of interest for the detection and the management of side effects as they would signal a risk of adverse effect prior to clinical signs and before any irreversible damage occurs. In this context, the nAPCsr could be used as a safety biomarker to predict the VTE risk induced by COC use, and to identify women for whom COC should not be initiated because of significant safety risks. This approach of proactively testing women for APCR before the initiation of a contraceptive therapy would allow precision medicine, as the goal would be to define the appropriate contraceptive therapy based on a biological measurement, i.e., the nAPCsr. Nevertheless, we could go even further and consider personalized medicine as the best way to prescribe the right treatment to the right person, should rely on available biological information (e.g., nAPCsr), as well as patient's personal preference, environmental and social factors.

The nAPCsr might also have the potential to become a surrogate. Indeed, for a biomarker to qualify as a surrogate, the biomarker must not only be correlated with the outcome, but the change in the biomarker must "explain" the change in the clinical outcome. The term "explains" invokes statistical inference, which can only be made with confidence if the observation is made in multiple therapies that all change the biomarker (Califf, 2018). Based on this statement, the correlation between nAPCsr and VTE risk has been demonstrated in our exploratory model (described in **Part II**), and changes in APCR levels explain changes in VTE risk as this observation has been made with multiple COCs.

Behind the surrogate endpoint denomination, there are 3 terms, the "candidate", the "reasonably likely" and the "validated" surrogate endpoints depending on clinical validation level. In our opinion, the nAPCsr might fall into the reasonably likely surrogate endpoint category, which is supported by strong mechanistic and/or epidemiologic rationale such that an effect on the surrogate endpoint is expected to be correlated with an endpoint intended to assess clinical benefit in clinical trial, but without sufficient clinical data to show that it is a validated surrogate endpoint (FDA-NIH Biomarker Working Group, 2020). In our case, the use of the nAPCsr as a surrogate endpoint is appealing because it would allow to dress far more rapidly and easily the safety haemostatic profile of new COC in clinical trials. However, to date, post-authorization safety studies remain essential. In this case, the recognition of the nAPCsr as a reasonably likely surrogate endpoint could help pharmaceutical companies developing new COCs to directly provide information about VTE risk before population-based phase 4 trials are completed.

In definitive, challenges in the coming years will be to promote the use of the nAPCsr in clinical laboratories to add the test to the analysis request form. The project is currently being initiated at the clinic CHC Mont Legia (Liège, Belgium) and discussions

are opened with hospitals in the other Wallonia provinces (Namur, Hainaut, and Brussels). At the same time, we will draft the dossier to be submitted to the EMA to obtain the biomarker qualification.

CONCLUSION

Throughout this thesis, we focused on hormone-induced coagulopathies and more specifically to the impact of COCs on the coagulation process. As healthcare professionals, we are concerned by iatrogenic effects of medicines and the birth control pill is one of the most widely used drugs worldwide. Moreover, as contraceptive therapies are administered to healthy young women with the aim to prevent unwanted pregnancies, the occurrence of side effects should be as low as possible and risk minimization strategies should be implemented accordingly.

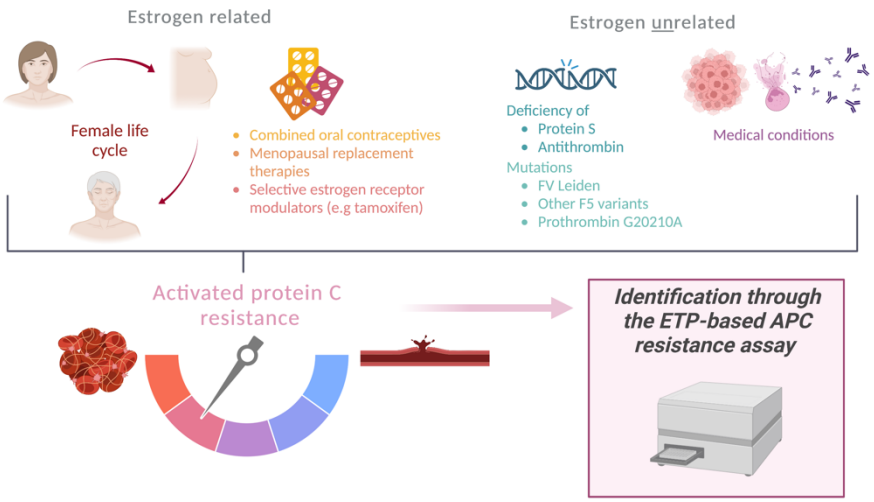
In this context, we have been working for the past 4 years in optimizing a global coagulation test. The ETP-based APCR assay was validated according to regulatory requirements and through this standardization, we have proposed a VTE risk prediction model for COC use. Indeed, the nAPCsr showed a strong correlation with VTE relative risk determined by long-term epidemiological studies, for several oestroprogestin associations. In the future, this model, if validated, could serve as a surrogate biomarker to avoid waiting almost a decade before positioning on the potential VTE risk of new COCs. In addition, it would be useful, on one hand, for individual VTE risk assessment before initiation of a contraceptive therapy and on the other hand, for identifying an abnormally high thrombotic risk in women already on the pill.

This thesis being dedicated of arguing strategies for minimizing the thrombotic risk associated with COCs, the recently commercialized E4-based product should obviously be part of the debate. Estetrol is a native oestrogen produced by the human foetus during pregnancy and has a pharmacological profile distinct from that of other oestrogens. Indeed, it is the first Native Oestrogen with Selective action in Tissues (NEST), acting as an agonist through ERα located in the nucleus but as an antagonist on the ERα-dependent MISS pathway. Through this specific pharmacodynamic profile, E4 has an agonist activity in the uterus, in the brain, and in bones; a mixed agonist/antagonist activity in the breast, and an almost neutral activity on the liver. The latter explains, at least in part, the lower impact of this new COC on haemostasis. Indeed, E4 associated with drospirenone showed a moderate increase of the nAPCsr compared to EE-containing COCs as well a non-clinically relevant increased thrombin generation compared to nonuser women. This is of paramount importance, as fears about COC continue to grow and this new birth

control pill could be a safe alternative for women wishing to use a contraception by the oral route, but reluctant with EE-containing COC use.

Using a combined oral contraceptive is not the only situation leading to a hormone-induced coagulopathy in women. When they stop their contraception and become pregnant, their thrombotic risk increases even more as the haemostatic balance changes to a hypercoagulable state to limit bleeding complications at delivery. In the continuum of life stage for women, the menopause happens, and the associated symptoms may lead to the use of menopausal replacement therapies, which are associated with an increased risk of VTE. Still related to hormones, oestrogen-dependent breast cancer is one of the most prevalent cancers in women and the use of tamoxifen has been identified as an additional risk factor for VTE in these patients. As a perspective, we obviously see an interest in using the nAPCsr to monitor the coagulability status of women, during these periods of their lives. Finally, the scope of this test goes beyond hormone-induced prothrombotic states and might be relevant in other pathologies such as haematological cancer and antiphospholipid syndrome.

In definitive, as the Chinese philosopher *Confucius* once said, "a *picture is worth a thousand words"*. As illustrated in the picture below, many situations (physiological, iatrogenic, or pathological) can lead to an APC resistance, representing an independent risk factor of VTE. This phenotype, regardless of the cause, may be detected by the ETP-based APCR assay, representing an opportunity to assess and score a hypercoagulable state, to help clinicians with decisions.



MEMENTO

THROMBIN GENERATION ASSAY

The thrombin generation assay (TGA) is performed on a Calibrated Automated Thrombogram (CAT), a system composed of a microplate fluorometer and a computer using the Thrombinoscope® software (version 5.0). Briefly the CAT system enables the recording of thrombin generation in plasma.

Citrated plasma samples are mixed with an activator reagent, STG-ThromboScreen® -TM, which contains a mixture of tissue factor (±5 pM) and phospholipids (±4 µM) in a 96-well plate. Coagulation then starts by adding FluCA kit, containing calcium chloride (160 nM) and a fluorogenic substrate (Z-Gly-Gly-Arg-AMC). Upon splitting by thrombin, the fluorescent AMC (7-amino-4-methylcoumarin) is released and measured with a 390 nm-excitation and a 460 nm-emission filter. For each plasma, the signal is corrected for substrate consumption, plasma colour variability and inner filter fluorescence effect by running in parallel calibrating wells in which plasma samples are mixed with the Thrombin Calibrator™. The Thrombinoscope computer software finally transforms the fluorescent signals into a quantity of thrombin generated over time, resulting in a thrombin generation curve named as Thrombogram (**Figure A**). This thrombogram reflects all the pro- and anticoagulant reactions that regulate both thrombin formation and inhibition, rendering the investigation of the initiation, the propagation, and the termination phase of the coagulation possible.

The following TGA parameters are extracted from the thrombogram:

- the lag-time (LT), corresponding to the start of thrombin generation (expressed in min
- the peak height (PH), corresponding to the maximal concentration of thrombin generated (expressed in nM)
- the time to peak (TTP), corresponding to the time to reach the peak (expressed in min)
- the endogenous thrombin potential (ETP), corresponding to the area under the curve (expressed in nM*min)
- the mean velocity rate index (mVRI), corresponding to the maximal rate of thrombin generation (expressed in nM/min).

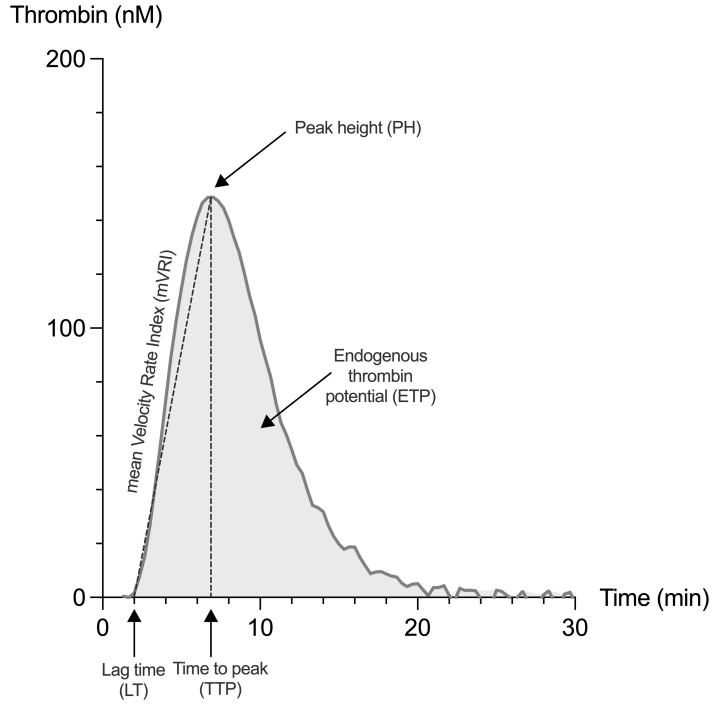


Figure A | Representation of a thrombin generation curve and associated parameters that are provided by the software analysing the thrombogram.

ETP-BASED APC RESISTANCE ASSAY

The ETP-based APC resistance (APCR) assay is based on the principle of thrombin generation assay (TGA). The activator reagent remains the STG-Thromboscreen® - TM, but a fixed concentration of activated protein C (APC) (Enzyme Research Laboratories, United Kingdom) is added for the APC-positive condition. The addition of APC enhances the APC-protein S anticoagulant complex in the tested plasma. The resulting effect is a reduction of the ETP, in comparison with the non-APC condition.

The quantity of APC to introduce in the test is defined at each batch change and targets a decrease of 90% of the ETP of a reference plasma, i.e., a healthy pooled plasma (HPP) (from men and women not using hormonal contraception, not carrier of FV Leiden or prothrombin G20210A mutations, and equally distributed).

This HPP is also used for the calculation of plasma samples nAPCsr (**Equation 2**). In presence of APC, the ETP-parameter of HPP decreases by 90% (blue curves in **Figure B**). On the other hand, in an APC-resistant plasma sample, typically observed in women using EE-containing products, thrombin generation in presence of APC does not decrease by 90% and thrombin generation in absence of APC is increased (pink curves in **Figure B**), compared to the HPP. On the nAPCsr scale, this transcribes into a value above 2.08, the upper limit of the normal range.

In practice, as the HPP is produced in small volumes, its use is restricted, so a commercial reference plasma must be used instead. As the use of the commercial reference plasma shows APCR compared to the healthy pooled plasma, we apply a correction factor for the plasma sample nAPCsr calculation (**Equation 9**).

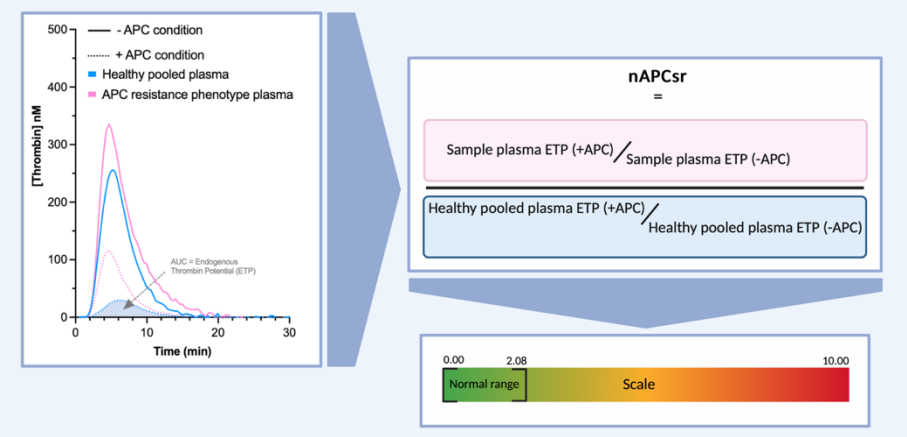


Figure B | Thrombin generation curves in absence (continuous line) and in presence of APC (dotted lines) of a healthy pooled plasma (blue) and of an APCR phenotype plasma (pink) along with the nAPCsr scale

EQUATIONS

Equation 1

APCsr (aPTT) = $\frac{sample\ aPTT\ (+APC)}{sample\ aPTT\ (-APC)}$

Equation 2

nAPCsr = $\frac{Sample\ plasma\ ETP\ (+APC) \Big/ Sample\ \ plasma\ ETP\ (-APC)}{Reference\ plasma\ \ ETP\ (+APC) \Big/ Reference\ plasma\ ETP\ (-APC)}$

Equation 3

Inhibition % = $1 - \frac{Sample\ ETP\ (+APC)}{Sample\ ETP\ (-APC)}, expressed\ in\ \%$

Equation 4

nAPCsr correction factor (F) =

$$\frac{Reference\ plasma\ ETP\ (+APC) \Big/ Reference\ plasma\ ETP\ (-APC)}{In\ house\ reference\ plasma\ ETP\ (+APC) \Big/ In\ house\ reference\ plasma\ ETP\ (-APC)}$$

Equation 5

ETP correction factor (CF) = $\frac{Commercial\ reference\ plasma\ ETP\ (-APC)}{In\ house\ reference\ plasma\ ETP\ (-APC)}$

Equation 6

Critical analytical difference (CD) $= 2.77\ \times\ estimated\ CV$

Equation 7

Critical SD = $\frac{CD}{100} \times\ inhibition\ percentage\ with\ current\ batch$

Equation 8

Acceptability range = $inhibition\ \%\ with\ current\ batch \pm SD\ critical$

Equation 9

Adjusted nAPCsr = $\frac{Sample\ ETP\ (+APC) \Big/ Sample\ ETP\ (-APC)}{STG{-}RefPlasma\ TS\ ETP\ (+APC) \Big/ STG{-}RefPlasma\ TS\ ETP\ (-APC)} * F$

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REFERENCES

Abdollahi, M., Cushman, M., & Rosendaal, F. (2003). Obesity: risk of venous thrombosis and the interaction with coagulation factor levels and oral contraceptive use. *Thromb Haemost*, *89*(3), 493-498.

Abot, A., Fontaine, C., Buscato, M., Solinhac, R., Flouriot, G., Fabre, A., Drougard, A., Rajan, S., Laine, M., Milon, A., Muller, I., Henrion, D., Adlanmerini, M., Valera, M. C., Gompel, A., Gerard, C., Pequeux, C., Mestdagt, M., Raymond-Letron, I., . . . Arnal, J. F. (2014). The uterine and vascular actions of estetrol delineate a distinctive profile of estrogen receptor alpha modulation, uncoupling nuclear and membrane activation. *EMBO Mol Med*, *6*(10), 1328-1346. https://doi.org/10.15252/emmm.201404112

Abou-Ismail, M. Y., Citla Sridhar, D., & Nayak, L. (2020). Estrogen and thrombosis: A bench to bedside review. *Thromb Res*, *192*, 40-51. https://doi.org/10.1016/j.thromres.2020.05.008

Agren, U. M., Anttila, M., Maenpaa-Liukko, K., Rantala, M. L., Rautiainen, H., Sommer, W. F., & Mommers, E. (2011). Effects of a monophasic combined oral contraceptive containing nomegestrol acetate and 17beta-oestradiol compared with one containing levonorgestrel and ethinylestradiol on haemostasis, lipids and carbohydrate metabolism. *Eur J Contracept Reprod Health Care*, *16*(6), 444-457. https://doi.org/10.3109/13625187.2011.604450

Ahrendt, H. J., Makalova, D., Parke, S., Mellinger, U., & Mansour, D. (2009). Bleeding pattern and cycle control with an estradiol-based oral contraceptive: a seven-cycle, randomized comparative trial of estradiol valerate/dienogest and ethinyl estradiol/levonorgestrel. *Contraception*, *80*(5), 436-444. https://doi.org/10.1016/j.contraception.2009.03.018

Aleksova, A., Di Nucci, M., Gobbo, M., Bevilacqua, E., Pradella, P., Salam, K., Barbati, G., De Luca, A., Mascaretti, L., & Sinagra, G. (2015). Factor-V HR2 haplotype and thromboembolic disease. *Acta Cardiol*, *70*(6), 707-711. https://doi.org/10.2143/AC.70.6.3120184

Apter, D., Zimmerman, Y., Beekman, L., Mawet, M., Maillard, C., Foidart, J. M., & Coelingh Bennink, H. J. (2016). Bleeding pattern and cycle control with estetrol-containing combined oral contraceptives: results from a phase II, randomised, dose-finding study (FIESTA). *Contraception*, *94*(4), 366-373. https://doi.org/10.1016/j.contraception.2016.04.015

Apter, D., Zimmerman, Y., Beekman, L., Mawet, M., Maillard, C., Foidart, J. M., & Coelingh Bennink, H. J. T. (2017). Estetrol combined with drospirenone: an oral contraceptive with high acceptability, user satisfaction, well-being and favourable body weight control. *Eur J Contracept Reprod Health Care*, *22*(4), 260-267. https://doi.org/10.1080/13625187.2017.1336532

Archer, D. F., Nakajima, S. T., Sawyer, A. T., Wentworth, J., Trupin, S., Koltun, W. D., Gilbert, R. D., & Ellman, H. (2013). Norethindrone acetate 1.0 milligram and ethinyl estradiol 10 micrograms as an ultra low-dose

oral contraceptive. *Obstet Gynecol*, *122*(3), 601-607. https://doi.org/10.1097/AOG.0b013e3182a1741c

Arnal, J. F., Lenfant, F., Metivier, R., Flouriot, G., Henrion, D., Adlanmerini, M., Fontaine, C., Gourdy, P., Chambon, P., Katzenellenbogen, B., & Katzenellenbogen, J. (2017). Membrane and Nuclear Estrogen Receptor Alpha Actions: From Tissue Specificity to Medical Implications. *Physiol Rev*, *97*(3), 1045-1087. https://doi.org/10.1152/physrev.00024.2016

Ay, C., Dunkler, D., Simanek, R., Thaler, J., Koder, S., Marosi, C., Zielinski, C., & Pabinger, I. (2011). Prediction of venous thromboembolism in patients with cancer by measuring thrombin generation: results from the Vienna Cancer and Thrombosis Study. *J Clin Oncol*, *29*(15), 2099-2103. https://doi.org/10.1200/JCO.2010.32.8294

Baglin, T., Gray, E., Greaves, M., Hunt, B. J., Keeling, D., Machin, S., Mackie, I., Makris, M., Nokes, T., Perry, D., Tait, R. C., Walker, I., & Watson, H. (2010). Clinical guidelines for testing for heritable thrombophilia. *Br J Haematol*, *149*(2), 209-220. https://doi.org/10.1111/j.1365-2141.2009.08022.x

Bagot, C. N., & Leishman, E. (2015). Establishing a reference range for thrombin generation using a standard plasma significantly improves assay precision. *Thromb Res*, *136*(1), 139-143. https://doi.org/10.1016/j.thromres.2015.04.020

Baker, S. G., & Kramer, B. S. (2020). Simple Methods for Evaluating 4 types of Biomarkers: Surrogate Endpoint, Prognostic, Predictive and Cancer Screening. *Biomarker Insights*, *15*, 1-8. https://doi.org/10.1177/1177271920946715

Bakker, E., Hendrikse, N. M., Ehmann, F., van der Meer, D. S., Llinares Garcia, J., Vetter, T., Starokozhko, V., & Mol, P. G. M. (2022). Biomarker Qualification at the European Medicines Agency: A Review of Biomarker Qualification Procedures From 2008 to 2020. *Clin Pharmacol Ther*, *112*(1), 69-80. https://doi.org/10.1002/cpt.2554

Bernardi, F. (2016). Better or worse than the original. *J Thromb Haemost*, *14*(7), 1350-1352. https://doi.org/10.1111/jth.13357

Bernardi, F., Faioni, E. M., Castoldi, E., Lunghi, B., Castaman, G., Sacchi, E., & Mannucci, P. M. (1997). A factor V Genetic Component Differing From Factor V R506Q Contributes to the Activated Protein C Resistance Phenotype. *Blood*, *90*, 1552-1557.

Bertina, R. M., Koeleman, B. P. C., Koster, T., Rosendaal, F., Dirven, R. J., de Ronde, H., van der Velden, P. A., & Reitsma, P. H. (1994). Mutation in blood coagulation factor V associated with resistance to activated protein C. *Nature*, *364*.

Blanco-Molina, M. A., Lozano, M., Cano, A., Cristobal, I., Pallardo, L. P., & Lete, I. (2012). Progestin-only contraception and venous thromboembolism. *Thromb Res*, *129*(5), e257-262. https://doi.org/10.1016/j.thromres.2012.02.042

Bloemenkamp, K., Rosendaal, F., Helmerhorst, F., Büller, H., & Vandenbroucke, J. P. (1995). Enhancement by factor V Leiden mutation of risk of deep-

vein thrombosis associated with oral contraceptives containing a thirg-generation progestogen. *The Lancet*, *346*, 1593-1596.

Blondon, M., Bodmer, A., Thouvenin, L., Lecompte, T., Righini, M., Fontana, P., & Casini, A. (2022). Differential impact of tamoxifen and aromatase inhibitors on thrombin generation: the prospective HEMOBREAST cohort. *Blood Adv*. https://doi.org/10.1182/bloodadvances.2021006623

Blondon, M., van Hylckama Vlieg, A., Wiggins, K. L., Harrington, L. B., McKnight, B., Rice, K. M., Rosendaal, F. R., Heckbert, S. R., Psaty, B. M., & Smith, N. L. (2014). Differential associations of oral estradiol and conjugated equine estrogen with hemostatic biomarkers. *J Thromb Haemost*, *12*(6), 879-886. https://doi.org/10.1111/jth.12560

Bonnar, J. (1987). Coagulation effects of oral contraception. *Am J Obstet Gynecol*, *157*(4 Pt 2), 1042-1048. https://doi.org/10.1016/s0002-9378(87)80129-1

Borgelt, L. M., & Martell, C. W. (2012). Estradiol valerate/dienogest: a novel combined oral contraceptive. *Clin Ther*, *34*(1), 37-55. https://doi.org/10.1016/j.clinthera.2011.11.006

Brenner, B. (2004). Haemostatic changes in pregnancy. *Thromb Res*, *114*(5-6), 409-414. https://doi.org/10.1016/j.thromres.2004.08.004

Brito, M. B., Ferriani, R. A., Meijers, J. C., Garcia, A. A., Quintana, S. M., Silva de Sa, M. F., & Vieira, C. S. (2012). Effects of the etonogestrel-releasing contraceptive implant inserted immediately postpartum on maternal hemostasis: a randomized controlled trial. *Thromb Res*, *130*(3), 355-360. https://doi.org/10.1016/j.thromres.2012.03.029

Brugge, J. M., Tans, G., Rosing, J., & Castoldi, E. (2006). Protein S levels modulate the activated protein C resistance phenotype induced by elevated prothrombin levels. *Thromb Haemost*, *95*(2), 236-242. https://doi.org/10.1160/TH05-08-0582

Califf, R. M. (2018). Biomarker definitions and their applications. *Exp Biol Med (Maywood)*, *243*(3), 213-221. https://doi.org/10.1177/1535370217750088

Canonico, M. (2015). Hormone therapy and risk of venous thromboembolism among postmenopausal women. *Maturitas*, *82*(3), 304-307. https://doi.org/10.1016/j.maturitas.2015.06.040

Canonico, M., Alhenc-Gelas, M., Plu-Bureau, G., Olie, V., & Scarabin, P. Y. (2010). Activated protein C resistance among postmenopausal women using transdermal estrogens: importance of progestogen. *Menopause*, *17*(6), 1122-1127. https://doi.org/10.1097/gme.0b013e3181e102eb

Castoldi, E., Brugge, J. M., Nicolaes, G. A., Girelli, D., Tans, G., & Rosing, J. (2004). Impaired APC cofactor activity of factor V plays a major role in the APC resistance associated with the factor V Leiden (R506Q) and R2 (H1299R) mutations. *Blood*, *103*(11), 4173-4179. https://doi.org/10.1182/blood-2003-10-3578

Castoldi, E., Hezard, N., Mourey, G., Wichapong, K., Poggi, M., Ibrahim-Kosta, M., Thomassen, L. G., Fournel, A., Hayward, C. P. M., Alessi, M. C., Hackeng, T. M., Rosing, J., & Morange, P. E. (2021). Severe

thrombophilia in a factor V-deficient patient homozygous for the Ala2086Asp mutation (FV Besancon). *J Thromb Haemost*, *19*(5), 1186-1199. https://doi.org/10.1111/jth.15274

Castoldi, E., & Rosing, J. (2010). APC resistance: biological basis and acquired influences. *J Thromb Haemost*, *8*(3), 445-453. https://doi.org/10.1111/j.1538-7836.2009.03711.x

CDC. (2016). *U.S. Medical Eligibility Criteria for COntraceptive Use, 2016*.

CeresPharma. (2021). *DHPC Louise*. https://www.afmps.be/sites/default/files/DHPC%20Louise%20FR%20-%20Website.pdf

Chiara Del Savio, M., De Fata, R., Facchinetti, F., & Grandi, G. (2020). Drospirenone 4 mg-only pill (DOP) in 24+4 regimen: a new option for oral contraception. *Expert Rev Clin Pharmacol*, *13*(7), 685-694. https://doi.org/10.1080/17512433.2020.1783247

Christin-Maitre, S., Laroche, E., & Bricaire, L. (2013). A new contraceptive pill containing 17b-estradiol and nomegestrol acetate. *Womens Health*, *9*(1), 13-23.

Clinical and Laboratory Standards Institute. (2010). Defining, Establishing, and Verifying Reference Intervals in the Clinical Laboratory; Approved Guideline — Third Edition. In *CLSI document EP28-A3C. Wayne, PA*: Clinical and Laboratory Standards Institute.

Clinical and Laboratory Standards Institute. (2013). User Evaluation of Between-Reagent Lot Variation. In *CLSI document EP26-A. Wayne, PA*: Clinical and Laboratory Standards Institute.

Clinical and Laboratory Standards Institute. (2016). Statistical Quality Control for Quantitative Measurement Procedures: Principles and Definitions. In *CLSI Guideline C24. Wayne, PA*: Clinical and Laboratory Standards Institute.

Coelingh Bennink, H. J., Holinka, C. F., & Diczfalusy, E. (2008). Estetrol review: profile and potential clinical applications. *Climacteric*, *11 Suppl 1*, 47-58. https://doi.org/10.1080/13697130802073425

Cosman, F., Baz-Hecht, M., Cushman, M., Vardy, M. D., Cruz, J. D., Nieves, J. W., Zion, M., & Lindsay, R. (2005). Short-term effects of estrogen, tamoxifen and raloxifene on hemostasis: a randomized-controlled study and review of the literature. *Thromb Res*, *116*(1), 1-13. https://doi.org/10.1016/j.thromres.2004.09.014

Cosmi, B., Legnani, C., Bernardi, F., Coccheri, S., & Palareti, G. (2003). Role of Family History in Identifying Women With Thrombophilia and Higher Risk of Venous Thromboembolism During Oral Contraception. *Arch Intern Med*, *163*.

Cramer, T. J., Griffin, J. H., & Gale, A. J. (2010). Factor V is an anticoagulant cofactor for activated protein C during inactivation of factor Va. *Pathophysiol Haemost Thromb*, *37*(1), 17-23. https://doi.org/10.1159/000315141

Creinin, M. D., & Jensen, J. T. (2020). Oral contraceptive generations - Time to stop using a marketing myth to define nomenclature. *Contraception*, *102*(3), 143-144. https://doi.org/10.1016/j.contraception.2020.05.017

Creinin, M. D., Westhoff, C. L., Bouchard, C., Chen, M. J., Jensen, J. T., Kaunitz, A. M., Achilles, S. L., Foidart, J. M., & Archer, D. F. (2021). Estetrol-drospirenone combination oral contraceptive: North American phase 3 efficacy and safety results. *Contraception*, *104*(3), 222-228. https://doi.org/10.1016/j.contraception.2021.05.002

Cronin-Fenton, D. P., Sondergaard, F., Pedersen, L. A., Fryzek, J. P., Cetin, K., Acquavella, J., Baron, J. A., & Sorensen, H. T. (2010). Hospitalisation for venous thromboembolism in cancer patients and the general population: a population-based cohort study in Denmark, 1997-2006. *Br J Cancer*, *103*(7), 947-953. https://doi.org/10.1038/sj.bjc.6605883

Cumming, A. M., Tait, R. C., Fildes, S., Yoong, A., Keeney, S., & Hay, C. R. M. (1995). Development of resistance to activated protein C during pregnancy. *British Journal of Haematology*, *90*, 725-727.

Curb, J., Prentice, R., Bray, P., Langer, R., Van Horn, L., Barnabei, V., Bloch, M., Cyr, M., Gass, M., Lepine, L., Rodabough, R., Sidney, S., Uwaifo, G., & Rosendaal, F. (2006). Venous Thrombosis and Conjugated Equine Estrogen in Women Without a Uterus. *Arch Intern Med*, *166*, 772-780.

Curvers, J., Christella, M., Thomassen, L. G., de Ronde, H., Bertina, R. M., Rosendaal, F. R., Tans, G., & Rosing, J. (2002). Effects of (pre-)analytical variables on activated protein C resistance determined via a thrombin generation-based assay. *Thromb Haemost*, *87*(3), 483-492. https://www.ncbi.nlm.nih.gov/pubmed/11924538

Curvers, J., Thomassen, M. C., Nicolaes, G. A., Van Oerle, R., Hamulyak, K., Hemker, H. C., Tans, G., & Rosing, J. (1999). Acquired APC resistance and oral contraceptives: differences between two functional tests. *Br J Haematol*, *105*(1), 88-94. https://www.ncbi.nlm.nih.gov/pubmed/10233368

Curvers, J., Thomassen, M. C., Rimmer, J., Hamulyak, K., van der Meer, J., Tans, G., Preston, F. E., & Rosing, J. (2002). Effects of hereditary and acquired risk factors of venous thrombosis on a thrombin generation-based APC resistance test. *Thromb Haemost*, *88*(1), 5-11. https://doi.org/10.1055/s-0037-1613145

Cushman, M., Costantino, J. P., Bovill, E. G., Wickerham, D. L., Buckley, L., Roberts, J. D., & Krag, D. N. (2003). Effect of tamoxifen on venous thrombosis risk factors in women without cancer: the Breast Cancer Prevention Tiral. *British Journal of Haematology*, *120*, 109-116.

Cushman, M., Kuller, L., Prentice, R., Rodabough, R., Psaty, B., Stafford, R., Sidney, S., & Rosendaal, F. (2004). Estrogen Plus Progestin and Risk of Venous Thrombosis. *JAMA*, *292*(13), 1573-1580.

Cuzick, J., Forbes, J. F., Sestak, I., Cawthorn, S., Hamed, H., Holli, K., Howell, A., & International Breast Cancer Intervention Study, I. I. (2007). Long-term results of tamoxifen prophylaxis for breast cancer--96-month follow-up of the randomized IBIS-I trial. *J Natl Cancer Inst*, *99*(4), 272-282. https://doi.org/10.1093/jnci/djk049

Dahlbäck, B. (1994). Physiological Anticoagulation : resistance to activated protein C and venous thromboembolism. *Journal of Clinical Investigation*, *94*.

Dahlbäck, B. (1997). Resistance to activated protein C caused by the R506Q mutation in the gene for factor V is a common risk factor for venous thrombosis. *J Intern Med Suppl*, *740*, 1-8. https://www.ncbi.nlm.nih.gov/pubmed/9350175

Dahlbäck, B. (2004). APC resistance: what have we learned since 1993? *J Lab Med*, *28*(1), 21-27.

Dahlbäck, B. (2005). The importance of the protein C system in the pathogenesis of venous thrombosis. *Hematology*, *10 Suppl 1*, 138-139. https://doi.org/10.1080/10245330512331390195

Dahlbäck, B., Carlsson, M., & Svensson, P. J. (1993). Familial thrombophilia due to a previously unrecognized mechanism characterized by poor anticoagulant response to activated protein C: prediction of a cofactor to activated protein C. *Proc. Natl. Acad. Sci.*, *90*, 1004-1008.

Dahlbäck, B., & Villoutreix, B. O. (2005). The anticoagulant protein C pathway. *FEBS Lett*, *579*(15), 3310-3316. https://doi.org/10.1016/j.febslet.2005.03.001

Dahm, A., Van Hylckama Vlieg, A., Bendz, B., Rosendaal, F., Bertina, R. M., & Sandset, P. M. (2003). Low levels of tissue factor pathway inhibitor (TFPI) increase the risk of venous thrombosis. *Blood*, *101*(11), 4387-4392. https://doi.org/10.1182/blood-2002-10-3188

Dargaud, Y., Luddington, R., Gray, E., Lecompte, T., Siegemund, T., Baglin, T., Hogwood, J., Regnault, V., Siegemund, A., & Negrier, C. (2010). Standardisation of thrombin generation test--which reference plasma for TGT? An international multicentre study. *Thromb Res*, *125*(4), 353-356. https://doi.org/10.1016/j.thromres.2009.11.012

Dargaud, Y., Luddington, R., Gray, E., Negrier, C., Lecompte, T., Petros, S., Hogwood, J., Bordet, J. C., Regnault, V., Siegemund, A., & Baglin, T. (2007). Effect of standardization and normalization on imprecision of calibrated automated thrombography: an international multicentre study. *Br J Haematol*, *139*(2), 303-309. https://doi.org/10.1111/j.1365-2141.2007.06785.x

Dargaud, Y., Wolberg, A. S., Luddington, R., Regnault, V., Spronk, H., Baglin, T., Lecompte, T., Ten Cate, H., & Negrier, C. (2012). Evaluation of a standardized protocol for thrombin generation measurement using the calibrated automated thrombogram: an international multicentre study. *Thromb Res*, *130*(6), 929-934. https://doi.org/10.1016/j.thromres.2012.07.017

de Bastos, M., Stegeman, B. H., Rosendaal, F. R., Van Hylckama Vlieg, A., Helmerhorst, F. M., Stijnen, T., & Dekkers, O. M. (2014). Combined oral contraceptives: venous thrombosis. *Cochrane Database Syst Rev*(3), CD010813. https://doi.org/10.1002/14651858.CD010813.pub2

de Visser, M. C., Guasch, J. F., Kamphuisen, P. W., Vos, H. L., Rosendaal, F. R., & Bertina, R. M. (2000). The HR2 haplotype of factor V: effects on factor V levels, normalized activated protein C sensitivity ratios and

the risk of venous thrombosis. *Thromb Haemost*, *83*(4), 577-582. https://www.ncbi.nlm.nih.gov/pubmed/10780320

de Visser, M. C., Rosendaal, F., & Bertina, R. M. (1999). A Reduced Sensitivity for Activated Protein C in the Absence of Factor V Leiden Increases the Risk of Venous Thrombosis. *Blood*, *93*(4), 1271-1276. https://doi.org/10.1182/blood.V93.4.1271

de Visser, M. C., van Hylckama Vlieg, A., Tans, G., Rosing, J., Dahm, A. E., Sandset, P. M., Rosendaal, F. R., & Bertina, R. M. (2005). Determinants of the APTT- and ETP-based APC sensitivity tests. *J Thromb Haemost*, *3*(7), 1488-1494. https://doi.org/10.1111/j.1538-7836.2005.01430.x

Dean, E., & Favaloro, E. J. (2020). The changing face of activated protein C resistance testing—a 10-year retrospective. *Annals of Blood*, *5*, 6-6. https://doi.org/10.21037/aob.2020.02.06

Demers, M., & Wagner, D. D. (2014). NETosis: a new factor in tumor progression and cancer-associated thrombosis. *Semin Thromb Hemost*, *40*(3), 277-283. https://doi.org/10.1055/s-0034-1370765

Devreese, K. M., Peerlinck, K., Arnout, J., & Hoylaerts, M. (2017). Laboratory detection of the antiphospholipid syndrome via calibrated automated thrombography. *Thrombosis and Haemostasis*, *101*(01), 185-196. https://doi.org/10.1160/th08-06-0393

Didembourg, M., Douxfils, J., Carlo, A., Mullier, F., Hardy, M., & Morimont, L. (2021). Effect of tissue factor pathway inhibitor on thrombin generation assay. *International Journal of Laboratory Hematology*. https://doi.org/10.1111/ijlh.13758

Dielis, A. W. J. H., Castoldi, E., Spronk, H. M. H., van Oerle, R., Hamulyak, K., Ten Cate, H., & Rosing, J. (2007). Coagulation factors and the protein C system as determinants of thrombin generation in a normal population. *Journal of thrombosis and haemostasis*, *6*, 125-131.

Dinger, J. (2020). COCs containing dienogest and 30 µg ethinylestradiol may carry a higher VTE risk compared to corresponding preparations with levonorgestrel: A meta-analysis of four large cohort studies. *Frontiers in Women's Health*, *5*(1). https://doi.org/10.15761/fwh.1000175

Dinger, J., Do Minh, T., & Heinemann, K. (2016). Impact of estrogen type on cardiovascular safety of combined oral contraceptives. *Contraception*, *94*(4), 328-339. https://doi.org/10.1016/j.contraception.2016.06.010

Douxfils, J., Gaspard, U., Taziaux, M., Jost, M., Bouvy, C., Lobo, R. A., Utian, W. H., & Foidart, J. M. (2022). Impact of estetrol (E4) on hemostasis, metabolism and bone turnover in postmenopausal women. *Climacteric*, 1-9. https://doi.org/10.1080/13697137.2022.2139599

Douxfils, J., Klipping, C., Duijkers, I., Kinet, V., Mawet, M., Maillard, C., Jost, M., Rosing, J., & Foidart, J. M. (2020). Evaluation of the effect of a new oral contraceptive containing estetrol and drospirenone on hemostasis parameters. *Contraception*, *102*(6), 396-402. https://doi.org/10.1016/j.contraception.2020.08.015

Douxfils, J., Morimont, L., & Bouvy, C. (2020). Oral Contraceptives and Venous Thromboembolism: Focus on Testing that May Enable Prediction and Assessment of the Risk. *Semin Thromb Hemost*, *46*(8), 872-886. https://doi.org/10.1055/s-0040-1714140

Douxfils, J., Morimont, L., Bouvy, C., de Saint-Hubert, M., Devalet, B., Devroye, C., Dincq, A. S., Dogne, J. M., Guldenpfennig, M., Baudar, J., Larock, A. S., Lessire, S., & Mullier, F. (2019). Assessment of the analytical performances and sample stability on ST Genesia system using the STG-DrugScreen application. *J Thromb Haemost*, *17*(8), 1273-1287. https://doi.org/10.1111/jth.14470

Douxfils, J., Morimont, L., Delvigne, A. S., Devel, P., Masereel, B., Haguet, H., Bouvy, C., & Dogne, J. M. (2020). Validation and standardization of the ETP-based activated protein C resistance test for the clinical investigation of steroid contraceptives in women: an unmet clinical and regulatory need. *Clin Chem Lab Med*, *58*(2), 294-305. https://doi.org/10.1515/cclm-2019-0471

Douxfils, J., Morimont, L., Gaspard, U., Utian, W. H., & Foidart, J. M. (2022). Estetrol is not a SERM but a NEST and has a specific safety profile on coagulation. *Thrombosis Research*. https://doi.org/10.1016/j.thromres.2022.09.007

Duarte, R. C. F., Ferreira, C. N., Rios, D. R. A., Reis, H. J. D., & Carvalho, M. D. G. (2017). Thrombin generation assays for global evaluation of the hemostatic system: perspectives and limitations. *Rev Bras Hematol Hemoter*, *39*(3), 259-265. https://doi.org/10.1016/j.bjhh.2017.03.009

Duchemin, J., Pan-Petesch, B., Arnaud, B., Blouch, M. T., & Abgrall, J. F. (2008). Influence of coagulation factors and tissue factor concentration on the thrombin generation test in plasma. *Thromb Haemost*, *99*(4), 767-773. https://doi.org/10.1160/TH07-09-0581

Duggan, C., Marriott, K., Edwards, R., & Cuzick, J. (2003). Inherited and acquired risk factors for venous thromboembolic disease among women taking tamoxifen to prevent breast cancer. *J Clin Oncol*, *21*(19), 3588-3593. https://doi.org/10.1200/JCO.2003.10.111

Eichinger, S., Hron, G., Kollars, M., & Kyrle, P. A. (2008). Prediction of recurrent venous thromboembolism by endogenous thrombin potential and D-dimer. *Clin Chem*, *54*(12), 2042-2048. https://doi.org/10.1373/clinchem.2008.112243

Eilertsen, A. L., Dahm, A. E. A., Hoibraaten, E., Lofthus, C. M., Mowinckel, M. C., & Sandset, P. M. (2019). Relationship between sex hormone binding globulin and blood coagulation in women on postmenopausal hormone treatment. *Blood Coagul Fibrinolysis*, *30*(1), 17-23. https://doi.org/10.1097/MBC.0000000000000784

Erman, M., Abali, H., Oran, B., Haznedaroglu, I. C., Canpinar, H., Kirazli, S., & Celik, I. (2004). Tamoxifen-induced tissue factor pathway inhibitor reduction: a clue for an acquired thrombophilic state? *Ann Oncol*, *15*(11), 1622-1626. https://doi.org/10.1093/annonc/mdh437

Eroglu, A., & Akar, N. (2011). Factor V Leiden, prothrombin G20210A and methylenetetrahydrofolate reductase (MTHFR) C677T

polymorphisms and the risk of tamoxifen-associated thromboembolism in breast cancer patients. *Thromb Res*, *127*(4), 384-385. https://doi.org/10.1016/j.thromres.2010.10.025

Eroglu, A., Cam, R., Yildiz, Z., & Akar, N. (2003). PT G20210A, factors V G1691A and 1299 His-Arg mutations and tamoxifen-associated thromboembolism in patients with breast cancer. *Thromb Res*, *111*(4-5), 317-319. https://doi.org/10.1016/j.thromres.2003.09.011

Esmon, C. T. (2003). The protein C pathway. *Chest*, *124*(3 Suppl), 26S-32S. https://doi.org/10.1378/chest.124.3_suppl.26s

European Medicines Agency. (1995). ICH Topic Q2(R1) - Validation of Analytical Procedures: Test and Methodology (CPMP/ICH/381/95). . http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500002662.pdf

European Medicines Agency. (2005). *Guideline on clinical investigation of steroid contraceptives in women - EMEA/CPMP/EWP/519/98 Rev 1*. https://www.ema.europa.eu/documents/scientific-guideline/guideline-clinical-investigation-steroid-contraceptives-women_en.pdf

European Medicines Agency. (2014). *Assessment report for combined hormonal contraceptives containing medicinal products-EMA/739865/2013*. https://www.ema.europa.eu/documents/referral/combined-hormonal-contraceptives-article-31-referral-prac-assessment-report_en.pdf

European Medicines Agency. (2021). *CHMP summary of positive opinion for Lydisilka*. https://www.ema.europa.eu/en/medicines/human/summaries-opinion/lydisilka

External quality Control for Assays and Tests With a focus on Thrombosis and Haemostasis. Thrombophilia - II: APC Resistance (without FV deficient plasma) - Survey 2021-M1 (A). Version 2.0.0. 14 June 2021.

Farmer, R. D. T., Lawrenson, R. A., Thompson, C. R., Kennedy, J. G., & Hambleton, I. R. (1997). Population-based study of risk of venous thromboembolism associated with various oral contraceptives. *The Lancet*, *349*(9045), 83-88. https://doi.org/10.1016/s0140-6736(96)07496-x

FDA-NIH Biomarker Working Group. (2020). *BEST (Biomarkers, EndpointS, and other Tools) Resource*.

Foidart, J. M. (2005). Added benefits of drospirenone for compliance. *Climacteric*, *8 Suppl 3*, 28-34. https://doi.org/10.1080/13697130500330309

Foidart, J. M., Gaspard, U., Pequeux, C., Jost, M., Gordenne, V., Tskitishvili, E., Gallez, A., Valera, M. C., Gourdy, P., Fontaine, C., Henrion, D., Genazzani, A. R., Lenfant, F., & Arnal, J. F. (2019). Unique Vascular Benefits of Estetrol, a Native Fetal Estrogen with Specific Actions in Tissues (NEST). In *Sex Steroids' Effects on Brain, Heart and Vessels* (pp. 169-195). https://doi.org/10.1007/978-3-030-11355-1_12

Food and Drug Administration. (2018). *Bioanalytical Method Validation - Guidance for Industry*. Retrieved 6 Feb from https://www.fda.gov/downloads/drugs/guidances/ucm070107.pdf

Fruzzetti, F., & Bitzer, J. (2010). Review of clinical experience with estradiol in combined oral contraceptives. *Contraception*, *81*(1), 8-15. https://doi.org/10.1016/j.contraception.2009.08.010

Fruzzetti, F., & Cagnacci, A. (2018). Venous thrombosis and hormonal contraception: what's new with estradiol-based hormonal contraceptives? *Open Access J Contracept*, *9*, 75-79. https://doi.org/10.2147/OAJC.S179673

Fruzzetti, F., Tremollieres, F., & Bitzer, J. (2012). An overview of the development of combined oral contraceptives containing estradiol: focus on estradiol valerate/dienogest. *Gynecol Endocrinol*, *28*(5), 400-408. https://doi.org/10.3109/09513590.2012.662547

FSRH. (2016). *UK medical eligibility criteria for contreceptive use (UKMEC)*.

Garber, J. E., Halabi, S., Tolaney, S. M., Kaplan, E., Archer, L., Atkins, J. N., Edge, S., Shapiro, C. L., Dressler, L., Paskett, E. D., Kimmick, G., Orcutt, J., Scalzo, A., Winer, E., Levine, E., Shahab, N., & Berliner, N. (2010). Factor V Leiden mutation and thromboembolism risk in women receiving adjuvant tamoxifen for breast cancer. *J Natl Cancer Inst*, *102*(13), 942-949. https://doi.org/10.1093/jnci/djq211

Gaspard, U., Taziaux, M., Jost, M., Coelingh Bennink, H., Utian, W. H., Lobo, R., & Foidart, J. M. (2022). A multicenter, randomized, placebo-controlled study to select the minimum effective dose of estetrol (E4) in postmenopausal participants (E4 Relief) : Part 2. Vaginal cytology, genitourinary syndrome of menopause and health-related quality of life. (Under Review). *Menopause*.

Gaussem, P., Alhenc-Gelas, M., Thomas, J. L., Bachelot-Loza, C., Remones, V., Ali, F. D., Aiach, M., & Scarabin, P. Y. (2011). Haemostatic effects of a new combined oral contraceptive, nomegestrol acetate/17beta-estradiol, compared with those of levonorgestrel/ethinyl estradiol. A double-blind, randomised study. *Thromb Haemost*, *105*(3), 560-567. https://doi.org/10.1160/TH10-05-0327

Gemzell-Danielsson, K., Sitruk-Ware, R., Creinin, M. D., Thomas, M., Barnhart, K. T., Creasy, G., Sussman, H., Alami, M., Burke, A. E., Weisberg, E., Fraser, I., Miranda, M. J., Gilliam, M., Liu, J., Carr, B. R., Plagianos, M., Roberts, K., & Blithe, D. (2019). Segesterone acetate/ethinyl estradiol 12-month contraceptive vaginal system safety evaluation. *Contraception*, *99*(6), 323-328. https://doi.org/10.1016/j.contraception.2019.02.001

Gerard, C., Arnal, J. F., Jost, M., Douxfils, J., Lenfant, F., Fontaine, C., Houtman, R., Archer, D. F., Reid, R. L., Lobo, R. A., Gaspard, U., Coelingh Bennink, H. J. T., Creinin, M. D., & Foidart, J. M. (2022). Profile of estetrol, a promising native estrogen for oral contraception and the relief of climacteric symptoms of menopause. *Expert Rev Clin Pharmacol*, *15*(2), 121-137. https://doi.org/10.1080/17512433.2022.2054413

Gérard, C., Mestdagt, M., Tskitishvili, E., Communal, L., Gompel, A., Silva, E., Arnal, J. F., Lenfant, F., Noel, A., Foidart, J. M., & Péqueux, C. (2015). Combined estrogenic and anti-estrogenic properties of estetrol on breast cancer may provide a safe therapeutic window for the treatment of menopausal symptoms. *Oncotarget*, *6*, 17621-17636.

Germain, M., Chasman, D. I., de Haan, H., Tang, W., Lindstrom, S., Weng, L. C., de Andrade, M., de Visser, M. C., Wiggins, K. L., Suchon, P., Saut, N., Smadja, D. M., Le Gal, G., van Hylckama Vlieg, A., Di Narzo, A., Hao, K., Nelson, C. P., Rocanin-Arjo, A., Folkersen, L., . . . Morange, P. E. (2015). Meta-analysis of 65,734 individuals identifies TSPAN15 and SLC44A2 as two susceptibility loci for venous thromboembolism. *Am J Hum Genet*, *96*(4), 532-542. https://doi.org/10.1016/j.ajhg.2015.01.019

Gessoni, G., & Valverde, S. (2007). Clinical evaluation of a functional prothrombin time-based assay for identification of factor V Leiden carriers in a group of Italian patients with venous thrombosis. *Blood Coagul Fibrinolysis*, *18*, 603-610.

Giesen, P. L. A., Gulpen, A. J. W., van Oerle, R., Ten Cate, H., Nagy, M., & Spronk, H. M. H. (2021). Calibrated automated thrombogram II: removing barriers for thrombin generation measurements. *Thromb J*, *19*(1), 60. https://doi.org/10.1186/s12959-021-00312-8

Graf, L. L., Welsh, C. H., Qamar, Z., & Marlar, R. A. (2003). Activated Protein C Resistance Assay Detects Thrombotic Risk Factors Other Than Factor V Leiden. *Am J Clin Pathol*, *119*, 52-60. https://doi.org/10.1092/QCUUNRMVJY8MWPPL

Grandi, G., Del Savio, M. C., Lopes da Silva-Filho, A., & Facchinetti, F. (2020). Estetrol (E4): the new estrogenic component of combined oral contraceptives. *Expert Rev Clin Pharmacol*, *13*(4), 327-330. https://doi.org/10.1080/17512433.2020.1750365

Griffin, J. H., Evatt, B., Wideman, C., & Fernandez, J. A. (1993). Anticoagulant proteinc C pathway in majority of thrombophilic patients. *Blood*, *82*, 1989-1993.

Hannaford, P. C. (2011). Epidemiology of the contraceptive pill and venous thromboembolism. *Thrombosis Research*, *127*, S30-S34. https://doi.org/10.1016/s0049-3848(11)70009-3

Hardy, M., Douxfils, J., Morimont, L., Didembourg, M., Carlo, A., de Maistre, E., Lecompte, T., & Mullier, F. (2021). Study of in vitro thrombin generation after neutralization of heparin. *International Journal of Laboratory Hematology*. https://doi.org/10.1111/ijlh.13703

Harrington, L. B., Blondon, M., Cushman, M., Kaunitz, A. M., Rossouw, J. E., Allison, M. A., Martin, L. W., Johnson, K. C., Rosing, J., Woods, N. F., LaCroix, A. Z., Heckbert, S. R., McKnight, B., & Smith, N. L. (2017). The cross-sectional association between vasomotor symptoms and hemostatic parameter levels in postmenopausal women. *Menopause*, *24*(4), 360-370. https://doi.org/10.1097/GME.0000000000000777

Heinemann, L. A. J. (1999). The changing scene - an unnecessary pill crisis. *Human reproduction update*, *5*, 746-755.

Heit, J. A., Kobbervig, C. E., James, A. H., Petterson, T. M., Bailey, K. R., & Melton III, L. J. (2005). Trends in the incidence of venous thromboembolism during pregnancy or postpartum: a 30-years-based study *Annals of Internal Medicine*, *143*, 697-706.

Hemker, H. C., Giesen, P., Al Dieri, R., Regnault, V., de Smedt, E., Wagenvoord, R., Lecompte, T., & Beguin, S. (2003). Calibrated automated thrombin generation measurement in clotting plasma. *Pathophysiol Haemost Thromb*, *33*(1), 4-15. https://doi.org/10.1159/000071636

Henkens, C. M., Bom, V. J., Seinen, A. J., & van der Meer, J. (1995). Sensitivity to activated protein C; influence of oral contraceptives and sex. *Thromb Haemost*, *73*(3), 402-404. https://www.ncbi.nlm.nih.gov/pubmed/7667823

Holinka, C. F., Diczfalusy, E., & Coelingh Bennink, H. J. (2008). Estetrol: a unique steroid in human pregnancy. *J Steroid Biochem Mol Biol*, *110*(1-2), 138-143. https://doi.org/10.1016/j.jsbmb.2008.03.027

Hotoleanu, C. (2017). Genetic Risk Factors in Venous Thromboembolism. *Adv Exp Med Biol*, *906*, 253-272. https://doi.org/10.1007/5584_2016_120

Hron, G., Kollars, N., & Binder, B. R. (2006). Identification Of Patients At Low Risk For Recurrent Venous Thromboembolism By Measuring Thrombin Generation. *Journal of Vascular Surgery*, *44*(6). https://doi.org/10.1016/j.jvs.2006.10.012

Hugon-Rodin, J., Alhenc-Gelas, M., Hemker, H. C., Brailly-Tabard, S., Guiochon-Mantel, A., Plu-Bureau, G., & Scarabin, P. Y. (2017). Sex hormone-binding globulin and thrombin generation in women using hormonal contraception. *Biomarkers*, *22*(1), 81-85. https://doi.org/10.1080/1354750X.2016.1204010

Hugon-Rodin, J., Horellou, M. H., Conard, J., Gompel, A., & Plu-Bureau, G. (2018). Type of Combined Contraceptives, Factor V Leiden Mutation and Risk of Venous Thromboembolism. *Thromb Haemost*, *118*(5), 922-928. https://doi.org/10.1055/s-0038-1641152

Inman, W. H. W., Vessey, M. P., Westerholm, B., & Engelund, A. (1970). Thromboembolic disease and the steroidal content of oral contraceptives : a report to the committee on safety of drugs. *British Medical Journal*, *2*, 209-209.

Institute, N. C. (2019). *Cancer Stat Facts: Female Breast Cancer*. Retrieved 2021 from https://seer.cancer.gov/statfacts/html/breast.html

Jackson, E., Curtis, K. M., & Gaffield, M. E. (2011). Risk of venous thromboembolism during the postpartum period: a systematic review. *Obstet Gynecol*, *117*(3), 691-703. https://doi.org/10.1097/AOG.0b013e31820ce2db

James, A. H. (2009). Pregnancy-associated thrombosis. *Hematology*, *277-285*.

Jenkins, P. V., Rawley, O., Smith, O. P., & O'Donnell, J. S. (2012). Elevated factor VIII levels and risk of venous thrombosis. *Br J Haematol*, *157*(6), 653-663. https://doi.org/10.1111/j.1365-2141.2012.09134.x

Jensen, J. T., Bitzer, J., & Serrani, M. (2013). Comparison of the pharmacologic and clinical profiles of new combined oral contraceptives containing

estradiol. *Open Access Journal of Contraception*. https://doi.org/10.2147/oajc.S50693

Jick, H., Jick, S., Gurewich, V., Myers, M. W., & Vasilakis, C. (1995). Risk of idiopathic cardiovascular death and nonfatal venous thromboembolism in women using oral contraceptives with differing progestagen components. *The Lancet*, *346*, 1589-1593.

Jilma-Stohlawetz, P., Lysy, K., Sunder-Plassmann, R., Belik, S., Jilma, B., Pabinger, I., & Quehenberger, P. (2021). Limitations of a calibrated, quantitative APC-R assay under routine conditions. *Int J Lab Hematol*, *43*(2), 318-323. https://doi.org/10.1111/ijlh.13378

Johnson, J. V., Lowell, J., Badger, G. J., Rosing, J., Tchaikovski, S., & Cushman, M. (2008). Effects of oral and transdermal hormonal contraception on vascular risk markers: a randomized controlled trial. *Obstet Gynecol*, *111*(2 Pt 1), 278-284. https://doi.org/10.1097/AOG.0b013e3181626d1b

Jordan, W. M., & Anand, J. K. (1961). Pulmonary embolism. *The Lancet*, *278*(7212), 1146-1147. https://doi.org/https://doi.org/10.1016/S0140-6736(61)91061-3

Junge, W., Mellinger, U., Parke, S., & Serrani, M. (2011). Metabolic and haemostatic effects of estradiol valerate/dienogest, a novel oral contraceptive: a randomized, open-label, single-centre study. *Clin Drug Investig*, *31*(8), 573-584. https://doi.org/10.2165/11590220-000000000-00000

Kadauke, S., Khor, B., & Van Cott, E. M. (2014). Activated protein C resistance testing for factor V Leiden. *Am J Hematol*, *89*(12), 1147-1150. https://doi.org/10.1002/ajh.23867

Kaur, S., Ali, A., Siahbalaei, Y., Ahmad, U., Pandey, A. K., & Singh, B. (2019). Could rs4379368 be a genetic marker for North Indian migraine patients with aura?: Preliminary evidence by a replication study. *Neurosci Lett*, *712*, 134482. https://doi.org/10.1016/j.neulet.2019.134482

Kemmeren, J. M., Algra, A., & Grobbee, D. E. (2001). Third generation oral contraceptives and risk of venous thrombosis: meta-analysis. *BMJ*, *323*(7305), 131-134. https://doi.org/10.1136/bmj.323.7305.131

Khan, S., & Dickerman, J. D. (2006). Hereditary thrombophilia. *Thromb J*, *4*, 15. https://doi.org/10.1186/1477-9560-4-15

Khialani, D., le Cessie, S., Lijfering, W. M., Cannegieter, S. C., Rosendaal, F. R., & van Hylckama Vlieg, A. (2020). The joint effect of genetic risk factors and different types of combined oral contraceptives on venous thrombosis risk. *Br J Haematol*, *191*(1), 90-97. https://doi.org/10.1111/bjh.16666

Khor, B., & Van Cott, E. M. (2009). Laboratory evaluation of hypercoagulability. *Clin Lab Med*, *29*(2), 339-366. https://doi.org/10.1016/j.cll.2009.03.002

Klipping, C., Duijkers, I., Parke, S., Mellinger, U., Serrani, M., & Junge, W. (2011). Hemostatic effects of a novel estradiol-based oral contraceptive: an open-label, randomized, crossover study of estradiol valerate/dienogest versus ethinylestradiol/levonorgestrel. *Drugs R*

D, *11*(2), 159-170. https://doi.org/10.2165/11591200-000000000-00000

Kluft, C., & Meijer, P. (2010). External quality assessment for thrombin generation tests: an exploration. *Semin Thromb Hemost*, *36*(7), 791-796. https://doi.org/10.1055/s-0030-1265296

Kopatz, W. F., Brinkman, H. J. M., & Meijers, J. C. M. (2018). Use of DOAC Stop for elimination of anticoagulants in the thrombin generation assay. *Thromb Res*, *170*, 97-101. https://doi.org/10.1016/j.thromres.2018.08.014

Koster, T., Rosendaal, F. R., de Ronde, H., Briët, E., Vandenbroucke, J. P., & Bertina, R. M. (1993). Venous thrombosis due to poor anticoagulant response to activated protein C-Leiden Thrombophilia Study. *The Lancet*, *342*.

Kuhl, H. (1996). Effects of progestogens on haemostasis. *Maturitas*, *24*, 1-19.

Kuhl, H. (2005). Pharmacology of estrogens and progestogens: influence of different routes of administration. *Climacteric*, *8 Suppl 1*, 3-63. https://doi.org/10.1080/13697130500148875

Kuhl, H. (2011). Pharmacology of progestogens *J Reproduktionsmed Endokrinol*, *8*(1), 157-176.

Kuhl, H., Bremser, H.-J., & Taubert, H.-D. (1982). Serum levels and pharmacokinetics of norethisterone after ingestion of lynestrenol: its relation to dose and stage of the menstrual cycle *Contraception*, *26*(3), 303-315.

Kuhnz, W., & Beier, S. (1994). Comparative progestational and androgenic activity of norgestimate and levonorgestrel in the rat. *Contraception*, *49*, 275-289.

Kujovich, J. L. (2004). Hormones and pregnancy: thromboembolic risks for women. *Br J Haematol*, *126*(4), 443-454. https://doi.org/10.1111/j.1365-2141.2004.05041.x

Kujovich, J. L. (2011). Factor V Leiden thrombophilia. *Genet Med*, *13*(1), 1-16. https://doi.org/10.1097/GIM.0b013e3181faa0f2

Lacroix, R., Judicone, C., Poncelet, P., Robert, S., Arnaud, L., Sampol, J., & Dignat-George, F. (2012). Impact of pre-analytical parameters on the measurement of circulating microparticles: towards standardization of protocol. *J Thromb Haemost*, *10*(3), 437-446. https://doi.org/10.1111/j.1538-7836.2011.04610.x

Lavigne-Lissalde, G., Sanchez, C., Castelli, C., Alonso, S., Mazoyer, E., Bal Dit Sollier, C., Drouet, L., Juhan-Vague, I., Gris, J. C., Alessi, M. C., & Morange, P. E. (2010). Prothrombin G20210A carriers the genetic mutation and a history of venous thrombosis contributes to thrombin generation independently of factor II plasma levels. *J Thromb Haemost*, *8*(5), 942-949. https://doi.org/10.1111/j.1538-7836.2010.03773.x

Lawrie, T. A., Helmerhorst, F. M., Maitra, N. K., Kulier, R., Bloemenkamp, K., & Gulmezoglu, A. M. (2011). Types of progestogens in combined oral contraception: effectiveness and side-effects. *Cochrane Database Syst Rev*(5), CD004861. https://doi.org/10.1002/14651858.CD004861.pub2

LeBlanc, E. S., & Laws, A. (1999). Benefits and risks of third-generaiton oral contraceptives. *Journal of general Internal Medicine*, *14*, 625-632.

Lebreton, A., Sinegre, T., Pereira, B., Lamblin, G., Duron, C., & Abergel, A. (2017). Plasma hypercoagulability in the presence of thrombomodulin but not of activated protein C in patients with cirrhosis. *J Gastroenterol Hepatol*, *32*(4), 916-924. https://doi.org/10.1111/jgh.13493

Lewis, M. A., Heinemann, L. A. J., Macrae, K. D., R., B., & Spitzer, W. O. (1996). The increased risk of venous thromboembolism and the use of third generation progestagens: role of bias in observational research. *Contraception*, *54*, 5-13.

Lidegaard, O., Lokkegaard, E., Svendsen, A. L., & Agger, C. (2009). Hormonal contraception and risk of venous thromboembolism: national follow-up study. *BMJ*, *339*(aug13 2), b2890. https://doi.org/10.1136/bmj.b2890

Lidegaard, O., Nielsen, L. H., Skovlund, C. W., Skjeldestad, F. E., & Lokkegaard, E. (2011). Risk of venous thromboembolism from use of oral contraceptives containing different progestogens and oestrogen doses: Danish cohort study, 2001-9. *BMJ*, *343*, d6423. https://doi.org/10.1136/bmj.d6423

Lin, H. F., Liao, K. F., Chang, C. M., Lin, C. L., Lai, S. W., & Hsu, C. Y. (2018). Correlation of the tamoxifen use with the increased risk of deep vein thrombosis and pulmonary embolism in elderly women with breast cancer: A case-control study. *Medicine (Baltimore)*, *97*(51), e12842. https://doi.org/10.1097/MD.0000000000012842

Lindberg, U., Crona, N., Stigendal, L., Teger-Nilsson, A., & G., S. (1989). A comparison between effects of estradiol valerate and low dose ethinyl estradiol on haemostasis parameters. *Thrombosis and Haemostasis*, *61*(1).

Lobo, R. A., & Stanczyk, F. A. (1994). New knowledge in the physiology of hormonal contraceptives. *Am J Obstet Gynecol*, *170*(5), 1499-1507.

Loeffen, R., Kleinegris, M. C., Loubele, S. T., Pluijmen, P. H., Fens, D., van Oerle, R., ten Cate, H., & Spronk, H. M. (2012). Preanalytic variables of thrombin generation: towards a standard procedure and validation of the method. *J Thromb Haemost*, *10*(12), 2544-2554. https://doi.org/10.1111/jth.12012

Lutsey, P. L., Folsom, A. R., Heckbert, S. R., & Cushman, M. (2009). Peak thrombin generation and subsequent venous thromboembolism: the Longitudinal Investigation of Thromboembolism Etiology (LITE) study. *J Thromb Haemost*, *7*(10), 1639-1648. https://doi.org/10.1111/j.1538-7836.2009.03561.x

Male, C., Mitchell, L., Julian, J., Vegh, P., Joshua, P., Adams, M., David, M., & Andrew, M. E. (2001). Acquired activated protein C resistance is associated with lupus anticoagulants and thrombotic events in pediatric patients with systemic lupus erythematosus. *Blood*, *97*, 844-849.

Mandala, M., & Tondini, C. (2012). Adjuvant therapy in breast cancer and venous thromboembolism. *Thromb Res*, *130 Suppl 1*, S66-70. https://doi.org/10.1016/j.thromres.2012.08.280

Mannucci, P. M., Bettega, D., Chantarangkul, V., Tripodi, A., Sacchini, V., & Veronesi, U. (1996). Effect of tamoxifen on measurements of hemostasis in healthy women. *Arch Intern Med*, *156*(16), 1806-1810. https://www.ncbi.nlm.nih.gov/pubmed/8790074

Mannucci, P. M., & Franchini, M. (2015). Classic thrombophilic gene variants. *Thromb Haemost*, *114*(5), 885-889. https://doi.org/10.1160/TH15-02-0141

Manyonda, I., V, S. T., Pirhadi, R., & Onwude, J. (2020). Progestogens are the problem in hormone replacement therapy: Time to reappraise their use. *Post Reprod Health*, *26*(1), 26-31. https://doi.org/10.1177/2053369119876490

Marco, A., Brocal, C., Martirena, F., Lucas, J., & Marco, P. (2012). Clinical and biological factors that contribute to thrombin generation in prothrombin G20210A carriers: a case-control study in a single Thrombophilia Center. *Thromb Res*, *129*(5), e266-268. https://doi.org/10.1016/j.thromres.2012.03.009

Mashchak, C. A., Lobo, R. A., Dozono-Takano, R., Eggena, P., Nakamura, R. M., Brenner, P. F., & Mishell, D. R. (1982). Comparison of pharmacodynamic properties of various estrogen formulations. *American Journal of Obstetrics and Gynecology*, *144*(5), 511-518. https://doi.org/10.1016/0002-9378(82)90218-6

Mathonnet, F., De Mazancourt, P., Bastenaire, B., Morot, M., Norbert, B., S., B., Borg, J.-Y., & Giudicelli, Y. (1996). Activated protein C sensitivity ratio in pregnant women at delivery. *British Journal of Haematology*, *92*, 244-246. https://doi.org/10.1046/j.1365-2141.1996.00271.x

Mawet, M., Maillard, C., Klipping, C., Zimmerman, Y., Foidart, J. M., & Coelingh Bennink, H. J. (2015). Unique effects on hepatic function, lipid metabolism, bone and growth endocrine parameters of estetrol in combined oral contraceptives. *Eur J Contracept Reprod Health Care*, *20*(6), 463-475. https://doi.org/10.3109/13625187.2015.1068934

McDaid, A., Logette, E., Buchillier, V., Muriset, M., Suchon, P., Pache, T. D., Tanackovic, G., Kutalik, Z., & Michaud, J. (2017). Risk prediction of developing venous thrombosis in combined oral contraceptive users. *PLoS One*, *12*(7), e0182041. https://doi.org/10.1371/journal.pone.0182041

Meade, T. W., Haines, A. P., North, W. R. S., Chakrabarti, R., Howarth, D. J., & Stirling, Y. (1977). Haemostatic, lipid, and blood-pressure profiles of women on oral contraceptives containing 50 µg or 30 µg oestrogen. *The Lancet*, *948-951*.

Medicines.org.uk. (2018). *Qlaira: Summary of Product Characyeristicz (SmPC) - (eMC)*. https://www.medicines.org.uk/emc/medicine/21700/SPC/Qlaira#gref

Michaud, J., & Tanackovic, G. (2016). About Pill Protect®. *Swiss Medical Weekly*. https://doi.org/10.4414/smw.2016.14392

Mohamed, A. B. O., Kelchtermans, H., Konings, J., van Daal, J., Al Marzouki, A., Harakeh, S., & de Laat, B. (2018). The effects of oral contraceptive usage on thrombin generation and activated protein C resistance in Saudi women, with a possible impact of the body mass index. *PLoS One*, *13*(10), e0206376. https://doi.org/10.1371/journal.pone.0206376

Monteyne, T., De Kesel, P., & Devreese, K. M. J. (2020). Interference of DOAC stop and DOAC remove in the thrombin generation assay and coagulation assays. *Thromb Res*, *192*, 96-99. https://doi.org/10.1016/j.thromres.2020.04.044

Moore, G. W. (2022). Snake Venoms in Diagnostic Hemostasis and Thrombosis. *Semin Thromb Hemost*, *48(2)*, 145-160. https://doi.org/10.1055/s-0041-1732465

Moore, G. W., Castoldi, E., Teruya, J., Morishita, E., & Adcock, D. M. (2022). Factor V Leiden-independent activated protein C resistance: Communication from the plasma coagulation inhibitors subcommittee of the International Society on Thrombosis and Haemostasis Scientific and Standardisation Committee. *J Thromb Haemost*, *21*(1), 164-174. https://doi.org/10.1016/j.jtha.2022.10.014

Moore, G. W., Van Cott, E. M., Cutler, J. A., Mitchell, M. J., & Adcock, D. M. (2019). Recommendations for clinical laboratory testing of activated protein C resistance; communication from the SSC of the ISTH. *J Thromb Haemost*, *17*(9), 1555-1561. https://doi.org/10.1111/jth.14532

Morange, P. E., Oudot-Mellakh, T., Cohen, W., Germain, M., Saut, N., Antoni, G., Alessi, M. C., Bertrand, M., Dupuy, A. M., Letenneur, L., Lathrop, M., Lopez, L. M., Lambert, J. C., Emmerich, J., Amouyel, P., & Tregouet, D. A. (2011). KNG1 Ile581Thr and susceptibility to venous thrombosis. *Blood*, *117*(13), 3692-3694. https://doi.org/10.1182/blood-2010-11-319053

Morange, P. E., & Tregouet, D. A. (2013). Current knowledge on the genetics of incident venous thrombosis. *J Thromb Haemost*, *11 Suppl 1*, 111-121. https://doi.org/10.1111/jth.12233

Morimont, L., Bouvy, C., Delvigne, A. S., Dogne, J. M., & Douxfils, J. (2020). Proof of concept of a new scale for the harmonization and the standardization of the ETP-based APC resistance. *J Thromb Haemost*, *18*(4), 895-904. https://doi.org/10.1111/jth.14745

Morimont, L., Dechamps, M., David, C., Bouvy, C., Gillot, C., Haguet, H., Favresse, J., Ronvaux, L., Candiracci, J., Herzog, M., Laterre, P. F., De Poortere, J., Horman, S., Beauloye, C., & Douxfils, J. (2022). NETosis and Nucleosome Biomarkers in Septic Shock and Critical COVID-19 Patients: An Observational Study. *Biomolecules*, *12*(8). https://doi.org/10.3390/biom12081038

Morimont, L., Didembourg, M., Haguet, H., Modaffari, E., Tillier, M., Bouvy, C., Lebreton, A., Dogné, J.-M., & Douxfils, J. (2021). Interlaboratory variability of activated protein C resistance using the ETP-based

APC resistance assay. *Research and Practice in Thrombosis and Haemostasis*, *5*(7). https://doi.org/10.1002/rth2.12612

Morimont, L., Dogné, J.-M., & Douxfils, J. (2020). Letter to the Editors-in-Chief in response to the article of Abou-Ismail, et al. entitled "Estrogen and thrombosis: A bench to bedside review" (Thrombosis Research 192 (2020) 40–51). *Thrombosis Research*, *193*, 221-223. https://doi.org/10.1016/j.thromres.2020.08.006

Morimont, L., Donis, N., Bouvy, C., Mullier, F., Dogne, J. M., & Douxfils, J. (2022). Laboratory Testing for the Evaluation of Phenotypic Activated Protein C Resistance. *Semin Thromb Hemost*. https://doi.org/10.1055/s-0042-1753511

Morimont, L., Haguet, H., Dogné, J.-M., Gaspard, U., & Douxfils, J. (2021). Combined Oral Contraceptives and Venous Thromboembolism: Review and Perspective to Mitigate the Risk. *Frontiers in Endocrinology*, *12*. https://doi.org/10.3389/fendo.2021.769187

Morimont, L., Leclercq, C., Didembourg, M., De Gottal, E., Carlo, A., Gaspard, U., Dogné, J.-M., & Douxfils, J. (2022). Analytical performance of the endogenous thrombin potential–based activated protein C resistance assay on the automated ST Genesia system. *Research and Practice in Thrombosis and Haemostasis*, *6*(3). https://doi.org/10.1002/rth2.12684

Negaard, H. F., Iversen, P. O., Ostenstad, B., Mowinckel, M. C., & Sandset, P. M. (2008). Increased acquired activated protein C resistance in unselected patients with hematological malignancies. *J Thromb Haemost*, *6*(9), 1482-1487. https://doi.org/10.1111/j.1538-7836.2008.03060.x

Negrier, C., Ninet, J., Bordet, J. C., Trzeciak, M., & Dargaud, Y. (2017). Use of calibrated automated thrombinography ± thrombomodulin to recognise the prothrombotic phenotype. *Thrombosis and Haemostasis*, *96*(11), 562-567. https://doi.org/10.1160/th06-03-0179

Nelson, A. L., Kaunitz, A. M., Kroll, R., Simon, J. A., Poindexter, A. N., Castano, P. M., Ackerman, R. T., Flood, L., Chiodo, J. A., 3rd, Garner, E. I., & Investigators, S. (2021). Efficacy, safety, and tolerability of a levonorgestrel/ethinyl estradiol transdermal delivery system: Phase 3 clinical trial results. *Contraception*, *103*(3), 137-143. https://doi.org/10.1016/j.contraception.2020.11.011

Nicolaes, G. A., Thomassen, M. C., Tans, G., Rosing, J., & Hemker, H. C. (1997). Effect of activated protein C on thrombin generation and on the thrombin potential in plasma of normal and APC-resistant individuals. *Blood Coagul Fibrinolysis*, *8*(1), 28-38. https://doi.org/10.1097/00001721-199701000-00006

Nogami, K., Shinozawa, K., Ogiwara, K., Matsumoto, T., Amano, K., Fukutake, K., & Shima, M. (2014). Novel FV mutation (W1920R, FVNara) associated with serious deep vein thrombosis and more potent APC resistance relative to FVLeiden. *Blood*, *123*(15), 2420-2428. https://doi.org/10.1182/blood-2013-10-530089

Nojima, J., Iwatani, Y., Ichihara, K., Tsuneoka, H., Ishikawa, T., Yanagihara, M., Takano, T., & Hidaka, Y. (2009). Acquired activated protein C resistance is associated with IgG antibodies to protein S in patients with systemic lupus erythematosus. *Thromb Res*, *124*(1), 127-131. https://doi.org/10.1016/j.thromres.2008.11.006

Nojima, J., Kuratsune, H., Suehisa, E., Iwatani, Y., & Kanakura, Y. (2005). Acquired activated protein C resistance associated with IgG antibodies against beta2-glycoprotein I and prothrombin as a strong risk factor for venous thromboembolism. *Clin Chem*, *51*(3), 545-552. https://doi.org/10.1373/clinchem.2004.043414

Norris, L. A., & Bonnar, J. (1996). The effect of oestrogen dose and progestogen type on haemostatic changes in women taking low dose oral contraceptives. *Br J Obstet Gynaecol*, *103*(3), 261-267. https://doi.org/10.1111/j.1471-0528.1996.tb09716.x

Norstrom, E., Thorelli, E., & Dahlback, B. (2002). Functional characterization of recombinant FV Hong Kong and FV Cambridge. *Blood*, *100*(2), 524-530. https://doi.org/10.1182/blood-2002-02-0343

Odlind, V., Milsom, I., Persson, I., & Victor, A. (2002). Can changes in sex hormone binding globulin predict the risk of venous thromboembolism with combined oral contraceptive pills? *Acta Obstet Gynecol Scand*, *81*, 482-490.

Olivieri, O., Friso, S., Manzato, F., Guella, A., Bernardi, F., Lunghi, B., Girelli, D., Azzini, M., Brocco, G., Russo, C., & Corrocher, R. (1995). Resistance to activated protein C in healthy women taking oral contraceptives. *Br J Haematol*, *91*(2), 465-470. https://doi.org/10.1111/j.1365-2141.1995.tb05323.x

Onitilo, A. A., Doi, S. A., Engel, J. M., Glurich, I., Johnson, J., & Berg, R. (2012). Clustering of venous thrombosis events at the start of tamoxifen therapy in breast cancer: a population-based experience. *Thromb Res*, *130*(1), 27-31. https://doi.org/10.1016/j.thromres.2011.11.025

Osterud, B., Robertsen, R., Asvang, G. B., & Thijssen, F. (1994). Resistance to activated protein C is reduced in women using oral contraceptives. *Blood Coagul Fibrinolysis*, *5*.

Pabinger, I., Thaler, J., & Ay, C. (2013). Biomarkers for prediction of venous thromboembolism in cancer. *Blood*, *122*(12), 2011-2018. https://doi.org/10.1182/blood-2013-04-460147

Palacios, S., Colli, E., & Regidor, P. A. (2019). Multicenter, phase III trials on the contraceptive efficacy, tolerability and safety of a new drospirenone-only pill. *Acta Obstet Gynecol Scand*, *98*(12), 1549-1557. https://doi.org/10.1111/aogs.13688

Patel, H. K., & Bihani, T. (2018). Selective estrogen receptor modulators (SERMs) and selective estrogen receptor degraders (SERDs) in cancer treatment. *Pharmacol Ther*, *186*, 1-24. https://doi.org/10.1016/j.pharmthera.2017.12.012

Perrin, J., Depasse, F., Lecompte, T., French-speaking, C. A. T. g., under the aegis of, G., French-speaking, C. A. T. g., & French-speaking, C. A. T. g. a. i. F. u. o. s. (2015). Large external quality assessment survey on

thrombin generation with CAT: further evidence for the usefulness of normalisation with an external reference plasma. *Thromb Res*, *136*(1), 125-130. https://doi.org/10.1016/j.thromres.2014.12.015

Peters, A. A., Buchanan, G., Ricciardelli, C., Bianco-Miotto, T., Centenera, M. M., Harris, J. M., Jindal, S., Segara, D., Jia, L., Moore, N. L., Henshall, S. M., Birrell, S. N., Coetzee, G. A., Sutherland, R. L., Butler, L. M., & Tilley, W. D. (2009). Androgen receptor inhibits estrogen receptor-alpha activity and is prognostic in breast cancer. *Cancer Res*, *69*(15), 6131-6140. https://doi.org/10.1158/0008-5472.CAN-09-0452

Pezeshkpoor, B., Castoldi, E., Mahler, A., Hanel, D., Muller, J., Hamedani, N. S., Biswas, A., Oldenburg, J., & Pavlova, A. (2016). Identification and functional characterization of a novel F5 mutation (Ala512Val, FVB onn) associated with activated protein C resistance. *J Thromb Haemost*, *14*(7), 1353-1363. https://doi.org/10.1111/jth.13339

Pomp, E. R., Lenselink, A. M., Rosendaal, F. R., & Doggen, C. J. (2008). Pregnancy, the postpartum period and prothrombotic defects: risk of venous thrombosis in the MEGA study. *J Thromb Haemost*, *6*(4), 632-637. https://doi.org/10.1111/j.1538-7836.2008.02921.x

Post, M. S., Christella, M., Thomassen, L. G., van der Mooren, M. J., van Baal, W. M., Rosing, J., Kenemans, P., & Stehouwer, C. D. (2003). Effect of oral and transdermal estrogen replacement therapy on hemostatic variables associated with venous thrombosis: a randomized, placebo-controlled study in postmenopausal women. *Arterioscler Thromb Vasc Biol*, *23*(6), 1116-1121. https://doi.org/10.1161/01.ATV.0000074146.36646.C8

Post, M. S., Rosing, J., Van Der Mooren, M. J., Zweegman, S., Van Baal, W. M., Kenemans, P., & Stehouwer, C. D. (2002). Increased resistance to activated protein C after short-term oral hormone replacement therapy in healthy post-menopausal women. *Br J Haematol*, *119*(4), 1017-1023. https://doi.org/10.1046/j.1365-2141.2002.03957.x

Post, M. S., van der Mooren, M. J., van Baal, W. M., Blankenstein, M. A., Merkus, H. M., Kroeks, M. V., Franke, H. R., Kenemans, P., & Stehouwer, C. D. (2003). Effects of low-dose oral and transdermal estrogen replacement therapy on hemostatic factors in healthy postmenopausal women: a randomized placebo-controlled study. *Am J Obstet Gynecol*, *189*(5), 1221-1227. https://doi.org/10.1067/s0002-9378(03)00599-4

Raps, M., Helmerhorst, F., Fleischer, K., Thomassen, S., Rosendaal, F., Rosing, J., Ballieux, B., & van Vliet, H. (2012). Sex hormone-binding globulin as a marker for the thrombotic risk of hormonal contraceptives. *J Thromb Haemost*, *10*(6), 992-997. https://doi.org/10.1111/j.1538-7836.2012.04720.x

Raps, M., Rosendaal, F., Ballieux, B., Rosing, J., Thomassen, S., Helmerhorst, F., & van Vliet, H. (2013). Resistance to APC and SHBG levels during use of a four-phasic oral contraceptive containing dienogest and estradiol valerate: a randomized controlled trial. *J Thromb Haemost*, *11*(5), 855-861. https://doi.org/10.1111/jth.12172

Reed, S., Koro, C., DiBello, J., Becker, K., Bauerfeind, A., Franke, C., & Heinemann, K. (2021). Prospective controlled cohort study on the safety of a monophasic oral contraceptive containing nomegestrol acetate (2.5mg) and 17beta-oestradiol (1.5mg) (PRO-E2 study): risk of venous and arterial thromboembolism. *Eur J Contracept Reprod Health Care*, *26*(6), 439-446. https://doi.org/10.1080/13625187.2021.1987410

Regidor, P. A. (2018). The clinical relevance of progestogens in hormonal contraception: present status and future developments. *Oncotarget*, *9*, 34628-34538.

Regnault, V., Béguin, S., Wahl, D., de Maistre, E., Coenraad Hemker, H., & Lecompte, T. (2003). Thrombinography shows acquired resistance to activated protein C in patients with lupus anticoagulants. *Thromb Haemost*, *89*(2), 208-212.

Richer and Mithra sign binding Heads of Terms for commercialization of Donesta. (20-12-2022). investors.mithra.com

Rodeghiero, F., & Tosetto, A. (1999). Activated protein C resistance and factor V Leiden mutation are independent risk factors for venous thromboembolism. *Ann Intern Med*, *130*, 643-650.

Rodgers, S. E., Wong, A., Gopal, R. D., Dale, B. J., Duncan, E. M., & McRae, S. J. (2014). Evaluation of pre-analytical variables in a commercial thrombin generation assay. *Thromb Res*, *134*(1), 160-164. https://doi.org/10.1016/j.thromres.2014.04.010

Rosendaal, F. R., Van Hylckama Vlieg, A., Tanis, B. C., & Helmerhorst, F. M. (2003). Estrogens, progestogens and thrombosis. *J Thromb Haemost*, *1*(7), 1371-1380. https://doi.org/10.1046/j.1538-7836.2003.00264.x

Rosenkranz, A., Hiden, M., Leschnik, B., Weiss, E. C., Schlembach, D., Lang, U., Gallistl, S., & Muntean, W. (2008). Calibrated automated thrombin generation in normal uncomplicated pregnancy. *Thromb Haemost*, *99*(2), 331-337. https://doi.org/10.1160/TH07-05-0359

Roshal, M., & Reyes Gil, M. (2019). Activated Partial Thromboplastin Time. In *Transfusion Medicine and Hemostasis* (pp. 779-781). https://doi.org/10.1016/b978-0-12-813726-0.00129-x

Rosing, J., Middeldorp, S., Curvers, J., Christella, M., Thomassen, L. G., Nicolaes, G. A., Meijers, J. C., Bouma, B. N., Buller, H. R., Prins, M. H., & Tans, G. (1999). Low-dose oral contraceptives and acquired resistance to activated protein C: a randomised cross-over study. *Lancet*, *354*(9195), 2036-2040. https://doi.org/10.1016/s0140-6736(99)06092-4

Rosing, J., Tans, G., Nicolaes, G. A., Thomassen, L. G., Van Oerle, R., Van der Ploeg, P. M. E. N., Heijnen, P., Hamulyak, K., & Hemker, H. C. (1997). Oral contraceptives and venous thrombosis: different sensitivities to activated protein C in women using second- and third-generation oral contraceptives. *British Journal of Haematology*, *97*.

Roullet, S., Labrouche, S., & Freyburger, G. (2019). Comparison of Two Thrombin Generation Methods, CAT and ST-Genesia, in Liver Transplant Patients. *Thromb Haemost*, *119*(6), 899-905. https://doi.org/10.1055/s-0039-1685452

Rovinski, D., Ramos, R. B., Fighera, T. M., Casanova, G. K., & Spritzer, P. M. (2018). Risk of venous thromboembolism events in postmenopausal women using oral versus non-oral hormone therapy: A systematic review and meta-analysis. *Thromb Res*, *168*, 83-95. https://doi.org/10.1016/j.thromres.2018.06.014

Ruan, X., Seeger, H., & Mueck, A. O. (2012). The pharmacology of nomegestrol acetate. *Maturitas*, *71*(4), 345-353. https://doi.org/10.1016/j.maturitas.2012.01.007

Ruhl, H., Schroder, L., Muller, J., Fimmers, R., Sukhitashvili, S., Welz, J., Kuhn, W. C., Oldenburg, J., Rudlowski, C., & Potzsch, B. (2014). Tamoxifen induces resistance to activated protein C. *Thromb Res*, *133*(5), 886-891. https://doi.org/10.1016/j.thromres.2014.02.004

Scarabin, P. Y., Hemker, H. C., Clement, C., Soisson, V., & Alhenc-Gelas, M. (2011). Increased thrombin generation among postmenopausal women using hormone therapy: importance of the route of estrogen administration and progestogens. *Menopause*, *18*(8), 873-879. https://doi.org/10.1097/gme.0b013e31820eee88

Schoni, R., Quehenberger, P., Wu, J. R., & Wilmer, M. (2007). Clinical evaluation of a new functional test for detection of activated protein C resistance (Pefakit APC-R Factor V Leiden) at two centers in Europe and the USA. *Thromb Res*, *119*(1), 17-26. https://doi.org/10.1016/j.thromres.2006.06.015

Segers, O., & Castoldi, E. (2009). Chapter 6 Factor V Leiden and activated protein C resistance. In (pp. 121-157). https://doi.org/10.1016/s0065-2423(09)49006-1

Sitruk-Ware, R. (2005). Pharmacology of different progestogens: the special case of drospirenone. *Climacteric*, *8 Suppl 3*, 4-12. https://doi.org/10.1080/13697130500330382

Sitruk-Ware, R. (2006). New progestagens for contraceptive use. *Hum Reprod Update*, *12*(2), 169-178. https://doi.org/10.1093/humupd/dmi046

Sitruk-Ware, R. (2008). Pharmacological profile of progestins. *Maturitas*, *61*(1-2), 151-157. https://doi.org/10.1016/j.maturitas.2008.11.011

Smith, N. L., Blondon, M., Wiggins, K. L., Harrington, L. B., van Hylckama Vlieg, A., Floyd, J. S., Hwang, M., Bis, J. C., McKnight, B., Rice, K. M., Lumley, T., Rosendaal, F. R., Heckbert, S. R., & Psaty, B. M. (2014). Lower risk of cardiovascular events in postmenopausal women taking oral estradiol compared with oral conjugated equine estrogens. *JAMA Intern Med*, *174*(1), 25-31. https://doi.org/10.1001/jamainternmed.2013.11074

Snoderly, H. T., Boone, B. A., & Bennewitz, M. F. (2019). Neutrophil extracellular traps in breast cancer and beyond: current perspectives on NET stimuli, thrombosis and metastasis, and clinical utility for diagnosis and treatment. *Breast Cancer Research*, *21*(1). https://doi.org/10.1186/s13058-019-1237-6

Speed, V., Roberts, L. N., Patel, J. P., & Arya, R. (2018). Venous thromboembolism and women's health. *Br J Haematol*, *183*(3), 346-363. https://doi.org/10.1111/bjh.15608

Spitzer, W. O. (1999). The aftermath of a pill scare: regression to reassurance. *Human reproduction update*, *5*, 736-745.

Spitzer, W. O., Lewis, M. A., Heinemann, L. A. J., Thorogood, M., & Macrae, K. D. (1996). Third generation oral contraceptives and risk of venous thromboembolic disorders: an international case-control study. *BMJ*, *312*.

Stadel, B. V. (1981). Oral contraceptives and cardiovascular disease (first of two parts). *New England Journal of Medicine*, *305*.

Steen, M., Norstrom, E. A., Tholander, A. L., Bolton-Maggs, P. H., Mumford, A., McVey, J. H., Tuddenham, E. G., & Dahlback, B. (2004). Functional characterization of factor V-Ile359Thr: a novel mutation associated with thrombosis. *Blood*, *103*(9), 3381-3387. https://doi.org/10.1182/blood-2003-06-2092

Stegeman, B. H., Helmerhorst, F. M., Vos, H. L., Rosendaal, F. R., & Van Hylckama Vlieg, A. (2012). Sex hormone-binding globulin levels are not causally related to venous thrombosis risk in women not using hormonal contraceptives. *J Thromb Haemost*, *10*(10), 2061-2067. https://doi.org/10.1111/j.1538-7836.2012.04878.x

Suchon, P., Al Frouh, F., Henneuse, A., Ibrahim, M., Brunet, D., Barthet, M. C., Aillaud, M. F., Venton, G., Alessi, M. C., Tregouet, D. A., & Morange, P. E. (2016). Risk factors for venous thromboembolism in women under combined oral contraceptive. The PILl Genetic RIsk Monitoring (PILGRIM) Study. *Thromb Haemost*, *115*(1), 135-142. https://doi.org/10.1160/TH15-01-0045

Suissa, S., Blais, L., Spitzer, W. O., Cusson, J., Lewis, M., & heinemann, L. (1997). First-time use of newer oral contraceptives and the risk of venous thromboembolism. *Contraception*, *56*, 141-146.

Svensson, P. J., & Dahlback, B. (1994). Resistance to activated protein C as a basis for venous thrombosis. *The New England Journal of Medicine*, *330*, 517-522.

Szecsi, P. B., Jorgensen, M., Klajnbard, A., Andersen, M. R., Colov, N. P., & Stender, S. (2010). Haemostatic reference intervals in pregnancy. *Thromb Haemost*, *103*(4), 718-727. https://doi.org/10.1160/TH09-10-0704

Szlam, F., Sreeram, G., Solomon, C., Levy, J. H., Molinaro, R. J., & Tanaka, K. A. (2011). Elevated factor VIII enhances thrombin generation in the presence of factor VIII-deficiency, factor XI-deficiency or fondaparinux. *Thromb Res*, *127*(2), 135-140. https://doi.org/10.1016/j.thromres.2010.10.017

Talon, L., Sinegre, T., Lecompte, T., Pereira, B., Massoulie, S., Abergel, A., & Lebreton, A. (2020). Hypercoagulability (thrombin generation) in patients with cirrhosis is detected with ST-Genesia. *J Thromb Haemost*, *18*(9), 2177-2190. https://doi.org/10.1111/jth.14963

Tans, G., van Hylckama Vlieg, A., Thomassen, M. C., Curvers, J., Bertina, R. M., Rosing, J., & Rosendaal, F. R. (2003). Activated protein C resistance determined with a thrombin generation-based test predicts for venous thrombosis in men and women. *Br J Haematol*, *122*(3), 465-470. https://doi.org/10.1046/j.1365-2141.2003.04443.x

Tchaikovski, S., Tans, G., & Rosing, J. (2006). Venous thrombosis and oral contraceptives: current status. *Womens Health (Lond)*, *2*(5), 761-772. https://doi.org/10.2217/17455057.2.5.761

Tchaikovski, S. N., Thomassen, M. C., Costa, S. D., Peeters, L. L., & Rosing, J. (2011). Role of protein S and tissue factor pathway inhibitor in the development of activated protein C resistance early in pregnancy in women with a history of preeclampsia. *Thromb Haemost*, *106*(5), 914-921. https://doi.org/10.1160/TH11-04-0244

Tchaikovski, S. N., van Vliet, H. A., Thomassen, M. C., Bertina, R. M., Rosendaal, F. R., Sandset, P. M., Helmerhorst, F. M., Tans, G., & Rosing, J. (2007). Effect of oral contraceptives on thrombin generation measured via calibrated automated thrombography. *Thromb Haemost*, *98*(6), 1350-1356. https://doi.org/10.1160/th07-07-0439

ten Kate, M. K., & van der Meer, J. (2008). Protein S deficiency: a clinical perspective. *Haemophilia*, *14*(6), 1222-1228. https://doi.org/10.1111/j.1365-2516.2008.01775.x

Tepper, N. K., Whiteman, M. K., Marchbanks, P. A., James, A. H., & Curtis, K. M. (2016). Progestin-only contraception and thromboembolism: A systematic review. *Contraception*, *94*(6), 678-700. https://doi.org/10.1016/j.contraception.2016.04.014

Thomassen, M., Bouwens, B. R. C., Wichapong, K., Suylen, D. P., Bouwman, F. G., Hackeng, T. M., & Koenen, R. R. (2023). Protein arginine deiminase 4 inactivates tissue factor pathway inhibitor-alpha by enzymatic modification of functional arginine residues. *J Thromb Haemost*. https://doi.org/10.1016/j.jtha.2023.01.017

Tripodi, A. (2016). Thrombin Generation Assay and Its Application in the Clinical Laboratory. *Clin Chem*, *62*(5), 699-707. https://doi.org/10.1373/clinchem.2015.248625

Tripodi, A., Chantarangkul, V., Negri, B., & Mannucci, P. M. (1998). Standardization of the APC resistance test. Effects of normalization of results by means of pooled normal plasma. *Thromb Haemost*, *79*(3), 564-566. https://www.ncbi.nlm.nih.gov/pubmed/9531041

Tripodi, A., Legnani, C., Chantarangkul, V., Cosmi, B., Palareti, G., & Mannucci, P. M. (2008). High thrombin generation measured in the presence of thrombomodulin is associated with an increased risk of recurrent venous thromboembolism. *J Thromb Haemost*, *6*(8), 1327-1333. https://doi.org/10.1111/j.1538-7836.2008.03018.x

Tripodi, A., Martinelli, I., Chantarangkul, V., Battaglioli, T., Clerici, M., & Mannucci, P. M. (2007). The endogenous thrombin potential and the risk of venous thromboembolism. *Thromb Res*, *121*(3), 353-359. https://doi.org/10.1016/j.thromres.2007.04.012

United Nations. (2019). *Contraceptive Use by Method 2019*. United Nations. https://www.un-ilibrary.org/content/books/9789210046527

Valera, M. C., Noirrit-Esclassan, E., Dupuis, M., Fontaine, C., Lenfant, F., Briaux, A., Cabou, C., Garcia, C., Lairez, O., Foidart, J. M., Payrastre, B., & Arnal, J. F. (2018). Effect of estetrol, a selective nuclear estrogen receptor modulator, in mouse models of arterial and venous thrombosis. *Mol*

Cell Endocrinol, *477*, 132-139. https://doi.org/10.1016/j.mce.2018.06.010

Van de Wouwer, M., Collen, D., & Conway, E. M. (2004). Thrombomodulin-protein C-EPCR system: integrated to regulate coagulation and inflammation. *Arterioscler Thromb Vasc Biol*, *24*(8), 1374-1383. https://doi.org/10.1161/01.ATV.0000134298.25489.92

van Hylckama Vlieg, A., Christiansen, S. C., Luddington, R., Cannegieter, S. C., Rosendaal, F. R., & Baglin, T. P. (2007). Elevated endogenous thrombin potential is associated with an increased risk of a first deep venous thrombosis but not with the risk of recurrence. *Br J Haematol*, *138*(6), 769-774. https://doi.org/10.1111/j.1365-2141.2007.06738.x

van Hylckama Vlieg, A., Helmerhorst, F. M., Vandenbroucke, J. P., Doggen, C. J., & Rosendaal, F. R. (2009). The venous thrombotic risk of oral contraceptives, effects of oestrogen dose and progestogen type: results of the MEGA case-control study. *BMJ*, *339*, b2921. https://doi.org/10.1136/bmj.b2921

Van Rooijen, M., Silveira, A., Hamsten, A., & Bremme, K. (2004). Sex hormone–binding globulin—A surrogate marker for the prothrombotic effects of combined oral contraceptives. *American Journal of Obstetrics and Gynecology*, *190*(2), 332-337. https://doi.org/10.1016/s0002-9378(03)00950-5

van Vliet, H. A., Frolich, M., Christella, M., Thomassen, L. G., Doggen, C. J., Rosendaal, F. R., Rosing, J., & Helmerhorst, F. M. (2005). Association between sex hormone-binding globulin levels and activated protein C resistance in explaining the risk of thrombosis in users of oral contraceptives containing different progestogens. *Hum Reprod*, *20*(2), 563-568. https://doi.org/10.1093/humrep/deh612

van Vliet, H. A., Winkel, T. A., Noort, I., Rosing, J., & Rosendaal, F. R. (2004). Prothrombotic changes in users of combined oral contraceptives containing drospirenone and cyproterone acetate. *J Thromb Haemost*, *2*(11), 2060-2062. https://doi.org/10.1111/j.1538-7836.2004.00983.x

Vandevelde, A., Chayoua, W., de Laat, B., Gris, J. C., Moore, G. W., Musial, J., Zuily, S., Wahl, D., & Devreese, K. M. J. (2022). Semiquantitative interpretation of anticardiolipin and antibeta2glycoprotein I antibodies measured with various analytical platforms: Communication from the ISTH SSC Subcommittee on Lupus Anticoagulant/Antiphospholipid Antibodies. *J Thromb Haemost*, *20*(2), 508-524. https://doi.org/10.1111/jth.15585

Velasco-Rodriguez, D., Laso, R. V., Garcia-Raso, A., Mahillo-Fernandez, I., Guzman-Lopez, K., Martin-Herrero, S., Barral, E. J., Vegas-Sanchez, M. D. C., Martinez-Becerra, M. J., de la Plaza, R., Romero, L. F. L., Minguez, D., Alonso-Dominguez, J. M., Lopez, C. B., Lopez, A. G., Fernandez, M. S. S., & Llamas-Sillero, P. (2020). Thrombin generation in subjects with lupus anticoagulant without prior thrombosis or gestational morbidities. *Thromb Res*, *196*, 425-431. https://doi.org/10.1016/j.thromres.2020.09.025

Vernon, E., Hiedemann, B., & Bowie, B. H. (2017). Economic Evaluations of Thrombophilia Screening Prior to Prescribing Combined Oral Contraceptives: A Systematic and Critical Review. *Appl Health Econ Health Policy*, *15*(5), 583-595. https://doi.org/10.1007/s40258-017-0318-x

Veronesi, U., Maisonneuve, P., Rotmensz, N., Bonanni, B., Boyle, P., Viale, G., Costa, A., Sacchini, V., Travaglini, R., D'Aiuto, G., Oliviero, P., Lovison, F., Gucciardo, G., del Turco, M. R., Muraca, M. G., Pizzichetta, M. A., Conforti, S., Decensi, A., & Italian Tamoxifen Study, G. (2007). Tamoxifen for the prevention of breast cancer: late results of the Italian Randomized Tamoxifen Prevention Trial among women with hysterectomy. *J Natl Cancer Inst*, *99*(9), 727-737. https://doi.org/10.1093/jnci/djk154

Vessey, M. P., & Inman, W. H. W. (1973). Speculation about mortality trends from venous thromboembolism disease in England and Wales and their relation to the pattern of oral contraceptive usage. *The journal of Obstetrics and Gynaecology of the British Commonwealth*, *80*, 562-566.

Vinogradova, Y., Coupland, C., & Hippisley-Cox, J. (2015). Use of combined oral contraceptives and risk of venous thromboembolism: nested case-control studies using the QResearch and CPRD databases. *BMJ*, *350*, h2135. https://doi.org/10.1136/bmj.h2135

Vinogradova, Y., Coupland, C., & Hippisley-Cox, J. (2019). Use of hormone replacement therapy and risk of venous thromboembolism: nested case-control studies using the QResearch and CPRD databases. *BMJ*, *364*, k4810. https://doi.org/10.1136/bmj.k4810

Waddington, A., Ferguson, C., & Reid, R. L. (2017). Contraception and Venous Thromboembolism: Risk Factors and Clinical Considerations. *Open Journal of Obstetrics and Gynecology*, *07*(01), 16-30. https://doi.org/10.4236/ojog.2017.71003

Wahl, D., Membre, A., Perret-Guillaume, C., Regnault, V., & Lecompte, T. (2009). Mechanisms of antiphospholipid-induced thrombosis: effects on the protein C system. *Current Rheumatology Reports*, *11*, 77-81.

Walker, A. J., Card, T. R., West, J., Crooks, C., & Grainge, M. J. (2013). Incidence of venous thromboembolism in patients with cancer - a cohort study using linked United Kingdom databases. *Eur J Cancer*, *49*(6), 1404-1413. https://doi.org/10.1016/j.ejca.2012.10.021

Walker, A. J., West, J., Card, T. R., Crooks, C., Kirwan, C. C., & Grainge, M. J. (2016). When are breast cancer patients at highest risk of venous thromboembolism? A cohort study using English health care data. *Blood*, *127*(7), 849-857; quiz 953. https://doi.org/10.1182/blood-2015-01-625582

Wessler, S., Gitel, S. N., Wan, L. S., & Pasternack, B. S. (1976). Estrogen-containing oral contraceptives agents : a basis for their thrombogenicity *JAMA*, *236*(19), 2179-2182.

Westhoff, C. L., Pike, M. C., Cremers, S., Eisenberger, A., Thomassen, S., & Rosing, J. (2017). Endogenous thrombin potential changes during the first

cycle of oral contraceptive use. *Contraception*, *95*(5), 456-463. https://doi.org/10.1016/j.contraception.2017.01.001

Wiegratz, I., & Kuhl, H. (2006). Metabolic and clinical effects of progestogens. *Eur J Contracept Reprod Health Care*, *11*(3), 153-161. https://doi.org/10.1080/13625180600772741

Wiegratz, I., Lee, J. H., Kutschera, E., Winkler, U. H., & Kuhl, H. (2004). Effect of four oral contraceptives on hemostatic parameters. *Contraception*, *70*(2), 97-106. https://doi.org/10.1016/j.contraception.2004.03.004

Wilmer, M., Stocker, C., Bühler, B., Conell, B., & Calatzis, A. (2004). Improved Distinction of Factor V Wild-Type and Factor V Leiden Using a Novel Prothrombin-Based Activated Protein C Resistance Assay. *American Journal of Clinical Pathology*, *122*(6), 836-842. https://doi.org/10.1309/t8avvh7qwgl0qtf5

World Health Organization. (2015). *Medical eligibility criteria for contraceptive use*

World Health Organization Collaborative Study of Cardiovascular Disease and Steroid Hormone Contraception. (1995). Effect of different progestagens in low oestrogen oral contraceptives on venous thromboembolic disease. *The Lancet*, *346*.

Xu, X., Chlebowski, R. T., Shi, J., Barac, A., & Haque, R. (2019). Aromatase inhibitor and tamoxifen use and the risk of venous thromboembolism in breast cancer survivors. *Breast Cancer Res Treat*, *174*(3), 785-794. https://doi.org/10.1007/s10549-018-05086-8

Yap, E. S., Timp, J. F., Flinterman, L. E., van Hylckama Vlieg, A., Rosendaal, F. R., Cannegieter, S. C., & Lijfering, W. M. (2015). Elevated levels of factor VIII and subsequent risk of all-cause mortality: results from the MEGA follow-up study. *J Thromb Haemost*, *13*(10), 1833-1842. https://doi.org/10.1111/jth.13071

Zeun, S., Lu, M., Uddin, A., Zeiler, B., Morrison, D., & Blode, H. (2009). Pharmacokinetics of an oral contraceptive containing oestradiol valerate and dienogest. *Eur J Contracept Reprod Health Care*, *14*(3), 221-232. https://doi.org/10.1080/13625180902850039

Zhang, D., & Trudeau, V. L. (2006). Integration of membrane and nuclear estrogen receptor signaling. *Comp Biochem Physiol A Mol Integr Physiol*, *144*(3), 306-315. https://doi.org/10.1016/j.cbpa.2006.01.025

Zuily, S., Aissa, K. A., Membre, A., Regnault, V., Lecompte, T., & Wahl, D. (2012). Thrombin generation in antiphospholipid syndrome. *Lupus*, *21*.

LIST OF PUBLICATIONS

Related to the thesis

1) Douxfils, J., Morimont, L., Delvigne, A. S., Devel, P., Masereel, B., Haguet, H., Bouvy, C., & Dogne, J. M. (2020). Validation and standardization of the ETP-based activated protein C resistance test for the clinical investigation of steroid contraceptives in women: an unmet clinical and regulatory need. *Clin Chem Lab Med*, *58*(2), 294-305. https://doi.org/10.1515/cclm-2019-0471
2) Morimont, L., Bouvy, C., Delvigne, A. S., Dogne, J. M., & Douxfils, J. (2020). Proof of concept of a new scale for the harmonization and the standardization of the ETP-based APC resistance. *J Thromb Haemost*, *18*(4), 895-904. https://doi.org/10.1111/jth.14745
3) Morimont, L., Dogné, J.-M., & Douxfils, J. (2020). Letter to the Editors-in-Chief in response to the article of Abou-Ismail, et al. entitled "Estrogen and thrombosis: A bench to bedside review" (Thrombosis Research 192 (2020) 40–51). *Thrombosis Research*, *193*, 221-223. https://doi.org/10.1016/j.thromres.2020.08.006
4) Douxfils, J., Morimont, L., & Bouvy, C. (2020). Oral Contraceptives and Venous Thromboembolism: Focus on Testing that May Enable Prediction and Assessment of the Risk. *Semin Thromb Hemost*, *46*(8), 872-886. https://doi.org/10.1055/s-0040-1714140
5) Morimont, L., Didembourg, M., Haguet, H., Modaffari, É., Tillier, M., Bouvy, C., Lebreton, A., Dogné, J. M., & Douxfils, J. (2021). Interlaboratory variability of activated protein C resistance using the ETP-based APC resistance assay. *Research and Practice in Thrombosis and Haemostasis*, *5*(7). https://doi.org/10.1002/rth2.12612
6) Morimont, L., Haguet, H., Dogné, J.-M., Gaspard, U., & Douxfils, J. (2021). Combined Oral Contraceptives and Venous Thromboembolism: Review and Perspective to Mitigate the Risk. *Frontiers in Endocrinology*, *12*. https://doi.org/10.3389/fendo.2021.769187
7) Morimont, L., Donis, N., Bouvy, C., Mullier, F., Dogne, J. M., & Douxfils, J. (2022). Laboratory Testing for the Evaluation of Phenotypic Activated Protein C Resistance. *Semin Thromb Hemost*. https://doi.org/10.1055/s-0042-1753511
8) Morimont, L., Gaspard, U., Foidart, J. M., Dogné, J.-M., & Douxfils, J. (2022). Contraceptifs oraux combinés et thrombo-embolie veineuse: quels sont vraiment les risques? *Medi-Sphere - L'hebdo du généraliste*, *701*, 39-49.
9) Morimont, L., Jost, M., Gaspard, U., Foidart, J. M., Dogne, J. M., & Douxfils, J. (2022). Low Thrombin Generation in Users of a Contraceptive Containing

Estetrol and Drospirenone. *J Clin Endocrinol Metab*. https://doi.org/10.1210/clinem/dgac511

10) Morimont, L., Leclercq, C., Didembourg, M., De Gottal, É., Carlo, A., Gaspard, U., Dogné, J. M., & Douxfils, J. (2022). Analytical performance of the endogenous thrombin potential–based activated protein C resistance assay on the automated ST Genesia system. *Research and Practice in Thrombosis and Haemostasis*, *6*(3). https://doi.org/10.1002/rth2.12684
11) Douxfils, J., Morimont, L., Gaspard, U., Utian, W. H., & Foidart, J.-M. (2022). Estetrol is not a SERM but a NEST and has a specific safety profile on coagulation. *Thrombosis Research*. https://doi.org/10.1016/j.thromres.2022.09.007
12) Morimont, L., Creinin, M. D., Gaspard, U., Foidart, J.-M., & Douxfils, J. (2023). Hormonal therapies and venous thrombosis: Estrogen matters! *Research and Practice in Thrombosis and Haemostasis*. https://doi.org/10.1016/j.rpth.2022.100021
13) Douxfils, J., Morimont, L., Creinin, M. D., Gaspard, U., & Foidart, J.-M. (2023). Hormonal therapies and venous thrombosis: considerations for prevention and management—a reappraisal. *Research and Practice in Thrombosis and Haemostasis*, *7*(4). https://doi.org/10.1016/j.rpth.2023.100155
14) Douxfils, J., Bouvy, C., & Morimont, L. (2023). Evaluation of Activated Protein C Resistance Using Thrombin Generation Test. *Methods Mol Biol*, *2663*, 211-224. https://doi.org/10.1007/978-1-0716-3175-1_12

Unrelated to the thesis

15) Douxfils, J., Morimont, L., Bouvy, C., de Saint-Hubert, M., Devalet, B., Devroye, C., Dincq, A. S., Dogne, J. M., Guldenpfennig, M., Baudar, J., Larock, A. S., Lessire, S., & Mullier, F. (2019). Assessment of the analytical performances and sample stability on ST Genesia system using the STG-DrugScreen application. *J Thromb Haemost*, *17*(8), 1273-1287. https://doi.org/10.1111/jth.14470
16) Reda, S., Morimont, L., Douxfils, J., & Ruhl, H. (2020). Can We Measure the Individual Prothrombotic or Prohemorrhagic Tendency by Global Coagulation Tests? *Hamostaseologie*, *40*(3), 364-378. https://doi.org/10.1055/a-1153-5824
17) Evrard, J., Siriez, R., Morimont, L., Themans, P., Laloy, J., Bouvy, C., Gheldof, D., Mullier, F., Dogne, J. M., & Douxfils, J. (2019). Optimal wavelength for the clot waveform analysis: Determination of the best resolution with minimal interference of the reagents. *Int J Lab Hematol*, *41*(3), 316-324. https://doi.org/10.1111/ijlh.12975
18) Evrard, J., Morimont, L., Benyahia, Z., Laloy, J., Siriez, R., Dogne, J. M., & Douxfils, J. (2020). Evaluation of the analytical performances of FibWave, a new sensitive

tool assessing the fibrin clot formation, to measure the effects of anticoagulants. *Thromb Res*, *191*, 78-81. https://doi.org/10.1016/j.thromres.2020.04.029

19) Evrard, J., Morimont, L., Didembourg, M., Haguet, H., Siriez, R., Dogne, J. M., & Douxfils, J. (2020). Assessment of acquired activated protein C resistance with the FibWave and comparison with the ETP-based APC resistance. *Int J Lab Hematol*. https://doi.org/10.1111/ijlh.13427
20) Didembourg, M., Douxfils, J., Mullier, F., Hardy, M., Favresse, J., & Morimont, L. (2021). Influence of C-reactive protein on thrombin generation assay. *Clin Chem Lab Med*. https://doi.org/10.1515/cclm-2020-1686
21) Didembourg, M., Douxfils, J., Carlo, A., Mullier, F., Hardy, M., & Morimont, L. (2021). Effect of tissue factor pathway inhibitor on thrombin generation assay. *International Journal of Laboratory Hematology*. https://doi.org/10.1111/ijlh.13758
22) Morimont, L., Dechamps, M., David, C., Bouvy, C., Gillot, C., Haguet, H., Favresse, J., Ronvaux, L., Candiracci, J., Herzog, M., Laterre, P.-F., De Poortere, J., Horman, S., Beauloye, C., & Douxfils, J. (2022). NETosis and Nucleosome Biomarkers in Septic Shock and Critical COVID-19 Patients: An Observational Study. *Biomolecules*, *12*(8). https://doi.org/10.3390/biom12081038
23) Bayart, J.-L., Morimont, L., Closset, M., Wieërs, G., Roy, T., Gerin, V., Elsen, M., Eucher, C., Van Eeckhoudt, S., Ausselet, N., David, C., Mullier, F., Dogné, J.-M., Favresse, J., & Douxfils, J. (2021). Confounding factors influencing the kinetics and magnitude of serological response following administration of NT162b2. *Microorganisms*, *9*. https://doi.org/10.3390/microorganisms10.3390/microorganisms9061340
24) Favresse, J., Gillot, C., Di Chiaro, L., Eucher, C., Elsen, M., Van Eeckhoudt, S., David, C., Morimont, L., Dogné, J.-M., & Douxfils, J. (2021). Neutralizing Antibodies in COVID-19 Patients and Vaccine Recipients after Two Doses of BNT162b2. *Viruses*, *13*(1364). https://doi.org/10.3390/v13071364
25) Favresse, J., Gillot, C., Oliveira, M., Cadrobbi, J., Elsen, M., Eucher, C., Laffineur, K., Rosseels, C., Van Eeckhoudt, S., Nicolas, J. B., Morimont, L., Dogne, J. M., & Douxfils, J. (2021). Head-to-Head Comparison of Rapid and Automated Antigen Detection Tests for the Diagnosis of SARS-CoV-2 Infection. *J Clin Med*, *10*(2). https://doi.org/10.3390/jcm10020265
26) Hardy, M., Douxfils, J., Morimont, L., Didembourg, M., Carlo, A., de Maistre, E., Lecompte, T., & Mullier, F. (2021). Study of in vitro thrombin generation after neutralization of heparin. *International Journal of Laboratory Hematology*. https://doi.org/10.1111/ijlh.13703
27) Dechamps, M., De Poortere, J., Martin, M., Gatto, L., Daumerie, A., Bouzin, C., Octave, M., Ginion, A., Robaux, V., Pirotton, L., Bodart, J., Gerard, L., Montiel, V., Campion, A., Gruson, D., Van Dievoet, M. A., Douxfils, J., Haguet, H., Morimont,

L., . . . Beauloye, C. (2021). Inflammation-Induced Coagulopathy Substantially Differs Between COVID-19 and Septic Shock: A Prospective Observational Study. *Front Med (Lausanne)*, *8*, 780750. https://doi.org/10.3389/fmed.2021.780750

28) Cabo, J., Morimont, L., Baudar, J., Guldenpfennig, M., Jacqmin, H., Soleimani, R., Lecompte, T., Douxfils, J., & Mullier, F. (2023). Variability among commercial batches of normal pooled plasma in lupus anticoagulant testing. *International Journal of Laboratory Hematology*. https://doi.org/10.1111/ijlh.13977

In submission

29) Didembourg M., Reda S., Oldenburg J., Rühl H., Douxfils J. Morimont L. Haemostatic imbalance induced by tamoxifen in oestrogen receptor-positive breast cancer patients: an observational study (in submission)