



**HAL**  
open science

## Monitoring of antivitamin K-dependent anticoagulation in rodents – Towards an evolution of the methodology to detect resistance in rodents

Sébastien Lefebvre, Claire Hascoët, Marlène Damin-Pernik, Benoit Rannou, Etienne Benoit, Virginie Lattard

### ► To cite this version:

Sébastien Lefebvre, Claire Hascoët, Marlène Damin-Pernik, Benoit Rannou, Etienne Benoit, et al.. Monitoring of antivitamin K-dependent anticoagulation in rodents – Towards an evolution of the methodology to detect resistance in rodents. *Pesticide Biochemistry and Physiology*, 2017, 138, pp.29 - 36. 10.1016/j.pestbp.2017.02.003 . hal-01571098

**HAL Id: hal-01571098**

**<https://hal.science/hal-01571098>**

Submitted on 1 Aug 2017

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Monitoring of antivitamin K-dependent anticoagulation in rodents – towards an evolution of the methodology to detect resistance in rodents

Sébastien Lefebvre, Claire Hascoët, Marlène Damin-Pernik, Benoit Rannou, Etienne Benoit, Virginie Lattard\*

USC 1233 INRA-Vetagro Sup, Veterinary School of Lyon, 1 avenue Bourgelat, 69280 Marcy l'Etoile, France

\* Corresponding author: Virginie Lattard

USC 1233 INRA-Vetagro Sup 69280 Marcy l'Etoile, France

Email: [virginie.lattard@vetagro-sup.fr](mailto:virginie.lattard@vetagro-sup.fr); Phone: +33-(0)478872727; Fax: +33-(0)478870516

Number of total words : 6163

## ABSTRACT

Vitamin K antagonists are used as rodenticides for pest control management. In rodents, prothrombin time is used to monitor their effect despite its limits and the emergence of many coagulation methods. The aim of this study is to explore different coagulation monitoring methods in order to propose the best method and the best parameter to monitor vitamin K antagonists effect in rodents.

The coagulation function was thus monitored with global coagulation assays and specialty assays after difethialone administration in rats.

Despite many parameters obtained by thromboelastometry, only clotting time and clot formation time obtained by ExTEM were modified. Their evolution was fast with doubling time

respectively of 4.0h and 3.7h but their increases were delayed with a lag time higher than 8h. Conversely, prothrombin time evolution presented a lag time of only 2h, but a higher doubling time of 7.2h. The measurements of factor VII and X activities were the most sensitive assays to monitor vitamin K antagonists effect with almost no lag time and the fastest evolution. Nevertheless, factor X was shown to be the only key factor driving prothrombin time. Monitoring factor X activity enables to follow most effectively the anticoagulation status in rats after rodenticides administration.

#### KEY-WORDS

vitamin K anticoagulants; intoxication monitoring; methods; clotting assay; thromboelastometry; clotting factor activity; rodents

#### ABBREVIATIONS

Activated Partial Thromboplastine time (aPTT) ; Clotting Time (CT) ; Clot formation time (CFT) ; carboxylated glutamate (Gla) ;extrinsic thromboelastometry (exTEM) ; glutamate (Glu) ; gamma-glutamyl carboxylase (GGCX) ; intrinsic thromboelastometry (inTEM) ; maximum clot firmness (MCF) ; Prothrombin time (PT) ; Thromboelastometry (TEM) ; Thrombodynamic potential index (TPI) ; Vitamin K antagonists (VKA)

#### CHEMICAL COMPOUNDS

Chemical compounds studied in this article: Difethialone (PubChem CID: 91771)

## 1 INTRODUCTION

For over half century, vitamin K antagonists (VKA) have been used in human medicine in prevention and treatment of thromboembolic disorders and as rodenticides for pest control

management. VKA acts as inhibitors of the vitamin K cycle. This cycle is a key component of the blood coagulation. Indeed, four clotting factors of the clotting cascade are vitamin K dependent – i.e. factor II, VII, IX and X. Their activity is conditioned by the gamma-carboxylation of their glutamate residues [1]. The gamma-carboxylation is performed in endoplasmic reticulum of hepatic cells by the enzyme gamma-glutamyl carboxylase with vitamin K as cofactors. For each gamma-carboxylation, vitamin K hydroquinone has to be converted in vitamin K epoxide. Then, vitamin K is regenerated by an enzyme, the Vitamin K epoxide reductase complex 1 (VKORC1). VKA are specific inhibitors of this enzyme [2]. This inhibition blocks the regeneration cycle, diminishing the vitamin K hydroquinone available for the gamma carboxylation of vitamin K dependent glutamate residues. Hence, the vitamin K dependent clotting factors are not functional, impairing the coagulation function.

VKA are used for pests control management because of their delayed action. This delayed action is crucial to success of the population management by avoiding the connection between these two events. Nevertheless, since the sixties, many cases of VKA resistances have been described [3–5]. Since this discovery, at least 25 different mutations of the *Vkorc1* gene have been described in Norway rats (*Rattus norvegicus*), some of these mutations lead to different levels of resistance against anticoagulant compounds [6]. Moreover, other resistance mechanisms have been described or suspected, some involving an overexpression of cytochromes P450 [7,8] and others with origins still unknown.

With the emergence of diverse resistance mechanisms to vitamin K antagonists in rodents, the diversity of VKA used for pests control management and the future development of new VKA, the need for proper coagulation testing has emerged. Initially, to evaluate resistance level, VKA efficiency tests were based on a survival challenge after a VKA administration. These challenges were quite long (many days) and have many shortcomings concerning the animal welfare and the repeatability. Later, new methods based on blood clotting response tests – i.e.,

the prothrombin time (PT), have been developed [9–11]. This new method reduce the experimentation time to 24 h, but is associated with major issues – i.e., the reproducibility and the repeatability of the test. Indeed, the PT measurement is widely dependent of the thromboplastin used. Thus, to compare the results the same reagent has to be used. In 2007, Prescott and al. have proposed to use the International Normalized Ratio (INR) in tests in order to increase the reproducibility [12]. However, this concern has not been completely solved by the use of International Normalized Ratio as reported by Prescott in [13]. Moreover, in human medicine, evolving clinical experience has made practitioners doubting the value of the PT test in the management of VKA anticoagulation [14–16]. Therefore, in human medicine, many coagulation tests have been explored to monitor VKA anticoagulation. Recent guidelines suggest the use of thromboelastometry (TEM) in the management of VKA anticoagulation [17]. With the diversity of blood clotting tests developed in human medicine, it might be today relevant to explore the interest of these tests to follow an anticoagulation by VKA in rodents to propose a better monitoring solution.

In this study, we monitored VKA anticoagulation by four clotting tests including PT and Activated Partial Thromboplastine time tests (aPTT) and TEM test with two different kinds of reagents. We completed this exploration by the measurement of the activity of the four vitamin K dependent clotting factors. Indeed, only gamma-carboxylated vitamin K dependent clotting factors are released in the blood of rats which allow their easy dosing [18]. Most of these blood clotting parameters have never been assessed or compared before in the perspective of the VKA-dependent coagulation studies. The aim of this study is to compare the kinetic evolution of different blood clotting parameters during the first 24h after the administration of a lethal dose of VKA and to study the possible correlation between the evolutions of these parameters. Our, result might be useful in order to refine the standardized efficiency test of VKA.

## 2 MATERIALS AND METHODS

### 2.1 ANIMALS

OFA-Sprague-Dawley rats of 8 weeks (250-300 g) were obtained from a commercial breeder (Charle Rivers, l'Arbresles, France) and acclimated for a minimal period of 5 days. The rats were housed four per cage under a constant photoperiod and ambient temperature. Experimental research was performed following international guidelines on animal welfare with approval from the ethics committee of the Veterinary School of Lyon. Animals were checked every day before difethialone administration and at least every 8 hours after, with a special attention to potential bleeding adverse events. In order to study the decay kinetics of the vitamin K-dependent blood clotting factors after administration of anticoagulants, rats (35 males) have been fed with vitamin K<sub>3</sub>-deficient food (Reference U8220 Version 231 from Scientific Animal Food and Engineering, Augy, France), at least 48 hours before the beginning and during the experience. To define the basal levels of blood parameters, a first group of 8 rats were killed 48h after the beginning of the vitamin K<sub>3</sub>-deficient feeding. Blood was collected in citrate tubes (3.2%; 1:9 v/v) by intracardiac taking and liver was collected and frozen at -80°C. The other animals received by force-feeding a *per os* administration of 10 mg/kg of difethialone (kindly provided by Liphatech) 48h after the beginning of the vitamin K<sub>3</sub>-deficient feeding, then a group of 3 rats was killed 1, 2, 4, 8, 10, 13, 16, 20 and 24 hours after the difethialone administration. Blood was collected in citrate tubes (3.2%; 1:9 v/v) by intracardiac taking and liver was collected and frozen at -80°C.

In order to explore the relation between the parameters, additional data were obtained with 33 male rats. They receive the same protocol at the beginning, they have been fed with vitamin K<sub>3</sub>-deficient food then they have received by force-feeding a *per os* administration of 10 mg/kg of difethialone in the same condition as the other rats. 48h after the difethialone administration

rats had received an oral dose of vitamin K ( $10\text{mg}\cdot\text{kg}^{-1}$ ). Then a group of 3 rats was killed at 135, 150, 165, 180, 210, 240, 270, 330, 360, 720 and 1440 minutes after the vitamin K administration. Blood was collected in citrate tubes (3.2%; 1:9 v/v) by intracardiac taking and liver was collected and frozen at  $-80^{\circ}\text{C}$ .

## 2.2 CLOTTING TIMES

Activated partial thromboplastin time (aPTT) and prothrombin time (PT) were measured using a STA Compact coagulation analyzer (Diagnostica Stago, Asnières, France) and Diagnostica Stago reagents. For aPTT determination,  $100\ \mu\text{L}$  of plasma were incubated at  $37^{\circ}\text{C}$  for 3 min with  $100\ \mu\text{L}$  of cephalin and activator (CK Prest, Stago), then  $100\ \mu\text{L}$  of  $0,025\ \text{M}$   $\text{CaCl}_2$  were added and the clotting time was recorded. For PT determination,  $100\ \mu\text{L}$  of plasma were added to  $200\ \mu\text{L}$  of tissue factor and calcium (Neoplastine, Stago) prewarmed to  $37^{\circ}\text{C}$  and the clotting time was recorded. The device automatically stopped if no clot appeared in 600s.

## 2.3 THROMBOELASTOMETRY

The measures of thromboelastometry are realized according to the protocol described by [19]. Analyses were performed using the thromboelastometer Rotem Delta (TEM international GmbH, Munich, Germany) and reagents provided by the manufacturer of the instrument. Those analyses were performed on whole citrated blood 30 minutes after blood collection according to recommendations. Briefly,  $300\ \mu\text{L}$  of prewarmed ( $37^{\circ}\text{C}$ ) citrated whole blood samples were added to clotting reagents in the cup. The analyses were then run for 60 minutes. Two types of clotting reagents were used to perform two profiles: intrinsic TEM (inTEMs reagent) to evaluate the intrinsic pathway (with activation by ellagic acid), and extrinsic TEM (exTEMs reagent) to evaluate the extrinsic pathway (with tissue factor activation). For these tests, the following parameters were recorded: clotting time (CT), the time until an amplitude of 2mm;

Clot formation time (CFT), the time between 2 mm amplitude and 20 mm amplitude; alpha-angle ( $\alpha$ ), angle between the baseline and a tangent to the clotting curve through the 2 mm point; maximum clot firmness (MCF), the maximum amplitude reached during the test. Furthermore, 1 parameter based on exTEM and InTEM was calculated: the thrombodynamic potential index (TPI) which shows thrombodynamic potential of the temogram, calculated from maximal clot elasticity (MCE;  $MCE = (100 \times MCF)/(100 - MCF)$ ) using the following equation:  $TPI = MCE/CFT$ .

#### 2.4 DETERMINATION OF CLOTTING FACTOR CONCENTRATIONS

Vitamin K-dependent clotting factors activities (II, VII, IX, X) were measured on citrated plasma using a chromogenic method on Konelab 30i (Thermoscientific) with Hyphen Biomed reagents.

More precisely, factor II was activated by incubation of citrate plasma with Echis Carinatus venom (0.25 mg/mL) in Tris-buffer (0,1mM Tris Hcl, pH 8.3 at 25°C, 0.2% BSA) at 37°C for 10 min. Thus, 50 µg of thrombin chromogenic substrate was added and absorbance was monitored at 405 nm for 1 min.

Factor VII activity was assayed by incubation of citrate plasma with calcium and tissue factor in excess to allow the formation of tenase complexes and addition of a saturating concentration of human factor X activated in factor Xa proportionally to the quantity of tenase complexes formed. Thus, activity of factor Xa was monitored at 405 nm for 2 min after the addition of a specific chromogenic substrate generating para-nitroaniline in the presence of factor Xa.

Factor IX activity was assayed by an initial incubation of thrombin, calcium, phospholipids and Factor XIa with citrate plasma. The addition of a second reagent allowed to add factor VIII:C and factor X in the mixture. Thus, 50 µg of thrombin chromogenic substrate was added and absorbance was monitored at 405 nm for 1 min.

Factor X was activated by the addition of Russel's viper venom. Thus, 50  $\mu$ g of thrombin chromogenic substrate was added and absorbance was monitored at 405 nm for 2 min.

## 2.5 STATISTICAL ANALYSIS AND MATHEMATICAL MODEL

Statistical analysis were performed with R (Version 3.1.2, R Foundation for Statistical Computing, Vienna, Austria). Results were expressed as mean values  $\pm$ SD or fitted value and confidence interval. For the evolution of each parameter against the time after the administration of difethialone, data were linearized with a logarithmic function. Then the slope of the regression curves was assessed with a Fisher test. The null hypothesis is that the slope is null.

For the evolution of each clotting factor against the prothrombin time, clotting factor and prothrombin time were linearized with a logarithmic function. Regression curves were compared on the slope and origin parameters with a Fisher test on the variance. The null hypothesis is that curves have the same slope or the same origin.

## 3 RESULTS

### 3.1 ASSESSMENT OF VKA-DEPENDENT HYPOCOAGULABILITY BY COAGULATION ASSAYS

VKA-dependent hypocoagulability was assessed during the first 24 hours following the administration of 10 mg/kg of difethialone by traditional coagulation tests – i.e. PT and Activated Partial Thromboplastin Time (aPTT) and by more global coagulation assay that is TEM using exTEM or inTEM reagents (Figure 1). To facilitate the analysis of each technique, results were thus represented comparatively to the corresponding results measured before difethialone administration then linearized with the natural logarithm function (Figure 2 and

Figure 3). The lag time before a significant modification of the parameter and the slope of parameter evolution after this lag time are resumed in table 1.

The four coagulation assays enabled to obtain a clotting time parameter (Figure 2). Only the clotting time obtained by TEM using the inTEM reagent did not significantly differ from basal level during the first 24 hours ( $P=0.693$ ). The others clotting time present a lag time ranged between 2 hours for PT method and 10 hours for TEM method using the exTEM, then significantly increase. The fastest increase was observed for the evolution of the clotting time obtained by TEM using the exTEM reagent with a slope of  $0.179 \text{ h}^{-1}$  ( $r^2=0.925$ ). The evolution of the clotting time obtained by PT and aPTT methods were slower with a slope of respectively  $0.096 \text{ h}^{-1}$  ( $r^2=0.972$ ) and  $0.030 \text{ h}^{-1}$  ( $r^2=0.825$ ).

In addition to the clotting time, four other parameters were assessed by TEM with exTEM or inTEM reagents. The evolution of CFT,  $\alpha$ , MCF and TPI are represented in Figure 3. No significant evolution was observed for any parameters obtained by TEM using the inTEM reagent during the 24h following difethialone administration. Concerning parameters obtained by TEM using the exTEM reagent only CFT increased significantly with a slope of  $0.187$  ( $r^2=0.819$ ) after a lag time of 8 hours.

### 3.2 ASSESSMENT OF VKA-DEPENDENT HYPOCOAGULABILITY BY DIRECT MEASUREMENT OF VITAMIN K-DEPENDENT CLOTTING FACTORS ACTIVITY

Activities of vitamin K-dependent clotting factors II, VII, IX and X were measured during the first 24 hours after difethialone administration (Figure 1). To facilitate the analysis, for each clotting factor activity, results are expressed comparatively to the corresponding clotting factor activity before difethialone administration then linearized with the natural logarithm function (Figure 4). A lag time was pinpointed only for factor IX and X, of respectively 2 hours and 1 hour. Conversely, one hour after the difethialone administration, prothrombin and factor VII

activities were already significantly decreased (respectively  $P=0.048$  and  $P=0.037$ ). Factor VII activity had the most important decrease with a slope of  $-0.26 \text{ h}^{-1}$  ( $r^2=0.978$ ), following by the factor X activity with a slope of  $-0.14 \text{ h}^{-1}$  ( $r^2=0.989$ ). Prothrombin and factor IX activity decreases were similar with a slope of respectively  $-0.07 \text{ h}^{-1}$  ( $r^2=0.937$ ) and  $-0.06 \text{ h}^{-1}$  ( $r^2=0.843$ ). Concerning the factor VII, its activity at 24h was under the limit of quantification. The lag time before a significant modification of the clotting factor activity and the slope of clotting factor activity evolution after this lag time are resumed in table 2.

### 3.3 ASSESSMENT OF THE CORRELATION BETWEEN COAGULABILITY PARAMETERS AND CLOTTING FACTORS

In order to evaluate the correlation between coagulability parameters and clotting factors evolution, we added data obtained from male rats which received a *per os* administration of  $10 \text{ mg.kg}^{-1}$  of difethialone, then 48 hours after an administration of  $10 \text{ mg.kg}^{-1}$  of vitamin K<sub>1</sub>. Owing to the important lag time observed with TEM parameters and the evolution weakness of the aPTT the correlation was successfully performed only with PT. Clotting factors and PT were expressed comparatively to the corresponding basal level before any administration, then transformed by natural logarithm function. Each vitamin K-dependent clotting factors evolution are represented in function of the PT in Figure 5. For each factor, slope and origin obtained after the linear regression of data during the decrease of clotting were compared with those obtained after the vitamin K<sub>1</sub> administration. Only the evolution of factor IX against PT was significantly different regarding slopes between decrease and regeneration phases ( $P=0.009$ ). Concerning prothrombin and factor VII, slopes between both phases were not significantly different (respectively  $P=0.150$  and  $P=0.952$ ) but their origins were different (respectively  $P<0.0001$  and  $P=0.007$ ). Finally, the linear regression parameters for the factor X evolution did not differ significantly between both phases for slopes ( $P=0.884$ ) or origins ( $P=0.793$ ).

## 4 DISCUSSION

In human medicine the prothrombin time is the main parameter of VKA therapy monitoring, in the form of the International Normalized Ratio form [20,21]. The aim of this monitoring is to avoid VKA adverse event and to follow possible change in patient physiology (hepatic induction). The purpose of the monitoring of parameters in rodent research is to test VKA molecules or rodent resistance strains [9,10]. They were standardized by the Rodenticide Resistance Action Committee in order to obtain a discriminating dose for each VKA based on the work of Prescott et al. [12,22]. Discriminating doses of anticoagulants, called ED50 (i.e., effective dose 50), were determined in rodents to characterize the susceptibility of rats to anticoagulants. ED50 corresponds to the dose leading to 50% of animals tested with a 5-fold increase of the International Normalized Ratio 24 hours after the administration of anticoagulants. International Normalized Ratio is calculated from PT. Consequently, in rodent VKA research, PT is also the key parameter too. There are some concerns about the use of ED50 method, on the one hand, the reproducibility as reported in [13], on the other hand, the number of animals used which may be important for this kind of test [23]. Consequently, it might be relevant to study other parameters in order to diminish the duration of the resistance test, with parameters which evolve quickly than prothrombin after AVK administration, and the number of animals used, with parameters which have less interindividual variability, and to improve the reproducibility of the method. Indeed, many techniques have been described assessing the blood coagulation in rats. Some of these techniques explore the blood capacity to coagulate. The simplest techniques as PT or aPTT give only a clotting time whereas the TEM using exTEM reagent – i.e. containing tissue factor - or inTEM reagent – i.e., containing ellagic acid or calcium - assess the global formation of the clot by providing many data. TEM is expected to become the future cornerstone in patient bleeding risk assessment [24]. It has been

yet used in some transfusion protocol and in cardiac and liver surgery [25,26] and recent studies try to increase its application scope [27]. In rats, TEM is increasingly used to measure coagulability in research [28,29]. Thus, it might help to refine ED50 test in rats by replacing INR. Finally, the last kind of methods explores the activity of the vitamin K dependent clotting factors themselves. All these tests were applied in this study in order to determinate the best method and the best parameter(s) to follow a VKA administration in rats and the possible correlation between clotting times and vitamin K dependent clotting factors.

To cause experimental anticoagulation in laboratory rats, we chose to administer difethialone orally. The oral administration corresponds to the normal route of administration of anticoagulant rodenticides in rodents. Difethialone is one of the most powerful anticoagulant molecules currently available. In addition, to be sure to have completely inhibited the vitamin K regeneration cycle, a dose 25 times higher than the ED50 was administered. The ED50 of difethialone for the male rats is of  $0.4 \text{ mg.kg}^{-1}$ , according to the technical monograph of 2003 [22]. This huge administration allows to determine the maximal evolution speed of clotting parameters following a VKA administration and thus to assess which of parameters has the best potential in the monitoring of vitamin K dependent coagulation. After administering the anticoagulant, coagulation was assessed by the various methods mentioned above. A good parameter to study of VKA efficiency has to be reliable and to vary quickly with the shortest lag time. Figure 6 presents the half-lives and doubling times resulting of the evolution of different parameters during the first 24 hours after administration of difethialone. The current reference assay, the PT, presented in our study a lag time of two hours, then a doubling time of 9.7 h with a good accuracy (0.972). As expected, aPTT evolved slower than PT. Conversely, two parameters determined by TEM performed with exTEM reagent, CT and CFT, seemed to change faster, after the lag time, than other clotting formation assessment parameters, and peculiarly than PT. Nevertheless, both parameters obtained by TEM using the ExTEM reagent

12

showed an important lag time before increasing; 10 hours for CT and 8 hours for the CFT. Moreover, doubling time of these parameters presented very wide confidence ranges. These large ranges were due, on the one hand, to a lack of reliability of this method in these conditions, and on the other hand, to the number of rats at each time used for the TEM method. Indeed, we respected the recommendations to perform thromboelastometry in rodents [19]. These recommendations impose time constraints in samples processing. Samples have to be ran within two hours following the sampling. Freezing the sample before the analysis is not possible, conversely to PT measurement where the sample can be stored at  $-70^{\circ}\text{C}$  until one month without significant differences [30]. In our case we had to adapt our protocol schedule and to restrict the measure to one specimen for each time for TEM methods. Another concern is about the quantity of blood needed which is of  $300\mu\text{L}$  against  $100\mu\text{L}$  of plasma for prothrombin time, making it difficult for its use in little rodent as mice, mainly if animal has to stay alive. Moreover, standardized method and reference values have not been defined for whole animals. Finally, the other parameters measured by TEM using exTEM reagent and all other parameters measured with inTEM reagent did not show significant evolution during the 24 hours following difethialone administration. Consequently, concerning methods which assess the blood capacity to coagulate and considering their lag time, their importance of evolution, their reliability and their simplicity of implementation in research schedule, PT is the best method to assess the effectiveness of VKA during the first 24 hours. This conclusion is in line with a previous study in human treated with warfarin [31].

Contrary to the parameters determined by the global coagulation tests, almost no lag time was observed for the evolution of clotting factors activities. Lag times for factor IX and X were very limited, respectively of 2 and 1 hour(s) and factors II and VII activities evolved immediately after VKA administration. Moreover, two of them, factors VII and X, evolved faster than the gold standard prothrombin time. Consequently, factors VII and X can be interesting to study

the kinetic and the efficiency of VKA in rodents, especially since they seem to be the subject of few interindividual variations. The great advantage of factor VII is its short half-life which may be useful to study the effect of VKA few hours after its administration. But 24 hours after the VKA administration, no more activity is detected for factor VII. Therefore 24h after VKA administration, factor X measurement should be preferred to monitor VKA efficiency. In addition to these benefits, clotting factors activities seem to be stable over storage time [32]. Moreover, the quantification of vitamin K dependent clotting factor activities is fairly simple in rats. Indeed, only gamma-carboxylated vitamin K dependent clotting factors are released in the blood of rats. However in other species of rodents such as mice, both factors, gamma- and non-gamma-carboxylated, are released. It could be an issue for prothrombin quantification. Indeed, chromogenic assay used to quantify the prothrombin doses gamma- and non-gamma-carboxylated forms of prothrombin. Consequently, the concentration of active prothrombin cannot be precisely determined in some species with this method. In order to quantify only active prothrombin, it would be necessary to purify prothrombin or to use a quantification by a prothrombin time based assay (the test plasma is completed by deficient II plasma) as used in human medicine [33]. Nevertheless this method has not been assessed for rodent use.

In order to use the measurement of vitamin K dependent clotting factors activity to monitor the VKA action instead of prothrombin time, it is essential to study the correlation between these parameters. In human medicine many studies have been performed to assess the importance of each clotting factor on prothrombin time by using dilution of clotting factors [34]. Moreover, many *in vitro* studies have been performed to characterize each reaction from the human coagulation cascade, which allows to model mathematically the cascade and to assess the importance of each factor [35,36]. However, concerning the rat, there is too little information on the cascade to create such model. But the study of the evolution of clotting factors relatively to prothrombin time might help to identify which clotting factor determine prothrombin time

during VKA intoxication because it is the restraining element. The difficulty of this analysis is that all vitamin K dependent decrease and so it is not possible to individualize the action of one. We hypothesized that if one vitamin K dependent clotting factor leads to the variation in the decrease of the clotting capacity, it might also lead to it in the restoration of this capacity. Thus for this clotting factor the curve obtained when clotting capacity decreased and that one obtained during the regeneration of clotting capacity might be the same. In our experiment, only the factor X fulfilled this requirement. This result is consistent with the hypothesis of Kerins and MacNicoll [37] that the factor X has greater effect on the prothrombin time of rats than others, whereas, in human, the dilution assays and the mathematical model results give prothrombin as the critical factor for the prothrombin time [36,38]. This relation between prothrombin time and factor X might be useful in order to refine ED50 test. Indeed, the activity of the factor X enables to monitor more efficiently a VKA-dependent anticoagulation in rodents than PT, while being totally correlated with the evolution of PT. Further investigations have to be conducted to confirm this opportunity by using lower dose of VKA. Nevertheless, the use of factor X activity measurement could thus diminish the duration of ED50 tests and improve the monitoring of VKA action in rodent research.

## 5 CONCLUSION

During the 24 hours following administration of an antivitamin K anticoagulant, Prothrombin time assay seems to be a better method than thromboelastometry to monitor coagulation in rodents. Nevertheless, measuring the activities of vitamin K-dependent clotting factors would enable to detect an anticoagulation status earlier. Table 3 summarizes the applicability of each technique to monitor VKA-anticoagulation in rodents and their benefits. Factor X is the critical factor which determine the prothrombin time in rats under a antivitamin K anticoagulant and enables to follow most effectively the anticoagulation status. Further studies should be

performed to confirm the relevance of this factor to follow the anticoagulation in the different strains of resistant rats.

## 6 ACKNOWLEDGEMENTS

This work was supported by grants ISI n°I1301001W “NEORAMUS” from Bpi France.

## 7 REFERENCES

- [1] J. Stenflo, P. Fernlund, W. Egan, P. Roepstorff, Vitamin K Dependent Modifications of Glutamic Acid Residues in Prothrombin, *Proc. Natl. Acad. Sci. U. S. A.* 71 (1974) 2730–2733. <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC388542/> (accessed July 20, 2015).
- [2] R. Lasseur, C. Longin-Sauvageon, B. Videmann, M. Billeret, P. Berny, E. Benoit, Warfarin resistance in a French strain of rats, *J. Biochem. Mol. Toxicol.* 19 (2006) 379–385. doi:10.1002/jbt.20104.
- [3] C.M. Boyle, Case of Apparent Resistance of *Rattus norvegicus* Berkenhout to Anticoagulant Poisons, *Nature.* 188 (1960) 517–517. doi:10.1038/188517a0.
- [4] J. Greaves, Warfarin-resistant rats, *R. Soc. Health J.* 90 (1970) 289. <http://www.ncbi.nlm.nih.gov/pubmed/5531174>.
- [5] J.H. Greaves, Resistance to anticoagulants in rodents, *Pestic. Sci.* 2 (1971) 276–279. doi:10.1002/ps.2780020609.
- [6] S. Rost, H.-J. Pelz, S. Menzel, A.D. MacNicoll, V. León, K.-J. Song, T. Jäkel, J. Oldenburg, C.R. Müller, Novel mutations in the VKORC1 gene of wild rats and mice – a response to 50 years of selection pressure by warfarin?, *BMC Genet.* 10 (2009) 4. doi:10.1186/1471-2156-10-4.
- [7] M. Ishizuka, F. Okajima, T. Tanikawa, H. Min, K.D. Tanaka, K.Q. Sakamoto, S. Fujita, Elevated Warfarin Metabolism in Warfarin-Resistant Roof Rats (*Rattus rattus*) in Tokyo, *Drug Metab. Dispos.* 35 (2007) 62–66. doi:10.1124/dmd.106.011775.
- [8] M.D. Markussen, A.-C. Heiberg, M. Fredholm, M. Kristensen, Differential expression of cytochrome P450 genes between bromadiolone-resistant and anticoagulant-susceptible Norway rats: a possible role for pharmacokinetics in bromadiolone resistance, *Pest Manag. Sci.* 64 (2008) 239–248. doi:10.1002/ps.1506.

- [9] J.E. Gill, G.M. Kerins, S.D. Langton, A.D. MacNicoll, Blood-Clotting Response Test for Bromadiolone Resistance in Norway Rats, *J. Wildl. Manage.* 58 (1994) 454–461. doi:10.2307/3809316.
- [10] J.E. Gill, G.M. Kerins, S.D. Langton, A.D. Macnicoll, The development of a blood clotting response test for discriminating between difenacoum-resistant and susceptible Norway rats (*Rattus norvegicus*, berk.), *Comp. Biochem. Physiol. Part C Comp. Pharmacol.* 104 (1993) 29–36. doi:10.1016/0742-8413(93)90106-U.
- [11] A.D. MacNicoll, J.E. Gill, Revised methodology for a blood-clotting response test for identification of warfarin-resistant Norway rats (*Rattus norvegicus*), *EPPO Bull.* 23 (1993) 701–707. doi:10.1111/j.1365-2338.1993.tb00571.x.
- [12] C. V. Prescott, A.P. Buckle, I. Hussain, S. Endepols, A standardised BCR resistance test for all anticoagulant rodenticides, <http://dx.doi.org/10.1080/09670870701245249>. (2007).
- [13] C. V. Prescott, R.A. Johnson, The laboratory evaluation of rodenticides., in: A.P. Buckle, R.H. Smith (Eds.), *Rodent Pests Their Control*, CABI, Wallingford, 2015: pp. 155–170. doi:10.1079/9781845938178.0155.
- [14] S. Sølbeck, S.R. Ostrowski, P.I. Johansson, A review of the clinical utility of INR to monitor and guide administration of prothrombin complex concentrate to orally anticoagulated patients., *Thromb. J.* 10 (2012) 5. doi:10.1186/1477-9560-10-5.
- [15] C.M. Jackson, M.P. Esnouf, Has the Time Arrived to Replace the Quick Prothrombin Time Test for Monitoring Oral Anticoagulant Therapy?, *Clin. Chem.* 51 (2005) 483–485. doi:10.1373/clinchem.2004.045393.
- [16] J.B. Segal, W.H. Dzik, Paucity of studies to support that abnormal coagulation test results predict bleeding in the setting of invasive procedures: an evidence-based review, *Transfusion.* 45 (2005) 1413–1425. doi:10.1111/j.1537-2995.2005.00546.x.

- [17] D.R. Spahn, B. Bouillon, V. Cerny, T.J. Coats, J. Duranteau, E. Fernández-Mondéjar, D. Filipescu, B.J. Hunt, R. Komadina, G. Nardi, E. Neugebauer, Y. Ozier, L. Riddez, A. Schultz, J.-L. Vincent, R. Rossaint, Management of bleeding and coagulopathy following major trauma: an updated European guideline., *Crit. Care.* 17 (2013) R76. doi:10.1186/cc12685.
- [18] T. Harauchi, K. Takano, M. Matsuura, T. Yoshizaki, Liver and Plasma Levels of Descarboxyprothrombin (PIVKA II) in Vitamin K Deficiency in Rats, *Jpn. J. Pharmacol.* 40 (1986) 491–499. doi:10.1254/jjp.40.491.
- [19] M. V. Wohlauer, E.E. Moore, J. Harr, E. Gonzalez, M. Frago, C.C. Silliman, A Standardized Technique for Performing Thrombelastography in Rodents, *Shock.* 36 (2011) 524–526. doi:10.1097/SHK.0b013e31822dc518.
- [20] J.D. Horton, B.M. Bushwick, Warfarin therapy: Evolving strategies in anticoagulation, *Am. Fam. Physician.* 59 (1999) 635–646.
- [21] M. Kuruvilla, C. Gurk-Turner, A review of warfarin dosing and monitoring, *Proc. (Bayl. Univ. Med. Cent).* 14 (2001) 305–306. <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1305837/> (accessed December 9, 2015).
- [22] Rodenticide Resistance Action Committee, A Reappraisal of Blood Clotting Response Tests for Anticoagulant Resistance and a proposal for a standardised BCR Test Methodology, CropLife international, 2003.
- [23] C. V Prescott, A.P. Buckle, Blood-clotting response tests for resistance to diphacinone and chlorophacinone in the Norway Rat (*Rattus norvegicus* Berk.), *Crop Prot.* 19 (2000) 291–296. doi:10.1016/S0261-2194(00)00018-1.
- [24] for the I.S. of T.M. and I. (SIMTI) W.P. Giancarlo Maria Liumbruno, Thromboelastography and thromboelastometry and the management of intra-operative transfusion therapy in massively bleeding patients, *Blood Transfus.* 10 (2012) 400.

doi:10.2450/2012.0130-11.

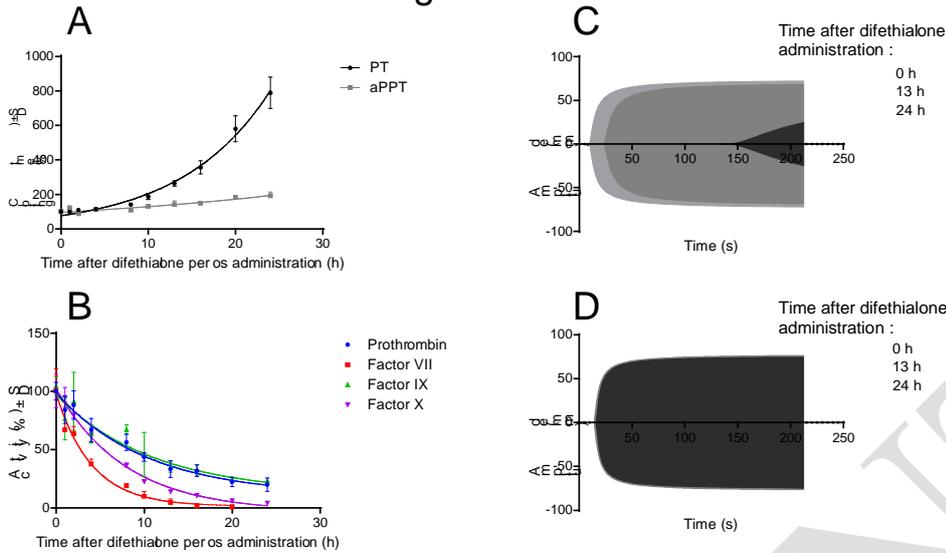
- [25] K. Gorlinger, D. Dirkmann, A.A. Hanke, M. Kamler, E. Kottenberg, M. Thielmann, H. Jakob, J. Peters, First-line therapy with coagulation factor concentrates combined with point-of-care coagulation testing is associated with decreased allogeneic blood transfusion in cardiovascular surgery: a retrospective, single-center cohort study, *Anesthesiology*. 115 (2011).
- [26] G. Bolt, T.D. Steenstrup, C. Kristensen, All post-translational modifications except propeptide cleavage are required for optimal secretion of coagulation factor VII, *Thromb. Haemost.* (2007). doi:10.1160/TH07-05-0332.
- [27] D.E. Schmidt, M. Holmström, A. Majeed, D. Näslin, H. Wallén, A. Ågren, Detection of elevated INR by thromboelastometry and thromboelastography in warfarin treated patients and healthy controls, *Thromb. Res.* 135 (2015) 1007–1011. doi:10.1016/j.thromres.2015.02.022.
- [28] L.N. Nielsen, B. Wiinberg, M. Häger, H.L. Holmberg, J.J. Hansen, K. Roepstorff, M. Tranholm, A novel *F8*<sup>-/-</sup> rat as a translational model of human hemophilia A, *J. Thromb. Haemost.* 12 (2014) 1274–1282. doi:10.1111/jth.12635.
- [29] I. Kampfmann, N. Bauer, S. Johannes, A. Moritz, Differences in hematologic variables in rats of the same strain but different origin, *Vet. Clin. Pathol.* 41 (2012) 228–234. doi:10.1111/j.1939-165X.2012.00427.x.
- [30] S. Alesci, M. Borggrefe, C.-E. Dempfle, Effect of freezing method and storage at –20 °C and –70 °C on prothrombin time, aPTT and plasma fibrinogen levels, *Thromb. Res.* 124 (2009) 121–126. doi:10.1016/j.thromres.2008.11.010.
- [31] C. Dunham, C. Rabel, B.M. Hileman, J. Schiraldi, E.A. Chance, M.T. Shima, A.A. Molinar, D.A. Hoffman, TEG® and RapidTEG® are unreliable for detecting warfarin-coagulopathy: a prospective cohort study, *Thromb. J.* 12 (2014) 4. doi:10.1186/1477-

9560-12-4.

- [32] M.A. Awad, T.E. Selim, F.A. Al-Sabbagh, Influence of storage time and temperature on international normalized ratio (INR) levels and plasma activities of vitamin K dependent clotting factors, *Hematology*. 9 (2004) 333–337. doi:10.1080/10245330400010646.
- [33] L.M. Baumann Kreuziger, Y.H. Datta, A.D. Johnson, N.D. Zantek, R. Shanley, M.T. Reding, Monitoring anticoagulation in patients with an unreliable prothrombin time/international normalized ratio: factor II versus chromogenic factor X testing., *Blood Coagul. Fibrinolysis*. 25 (2014) 232–6. doi:10.1097/MBC.0000000000000030.
- [34] P. Plum, Relation between Prothrombin Concentration and Clotting Time<sup>1</sup>, *Acta Med. Scand*. 115 (1943) 41–56. doi:10.1111/j.0954-6820.1943.tb15042.x.
- [35] E.A. Shavlyugin, L.G. Hanin, M.A. Khanin, Dynamics of pathologic clot formation: A mathematical model, *J. Theor. Biol.* 340 (2014) 96–104. doi:10.1016/j.jtbi.2013.08.007.
- [36] M.A. Khanin, D. V Rakov, A.E. Kogan, Mathematical Model for the Blood Coagulation Prothrombin Time Test, *Thromb. Res.* 89 (1998) 227–232. doi:10.1016/S0049-3848(97)00288-0.
- [37] G.M. Kerins, A.D. MacNicoll, Comparison of the half-lives and regeneration rates of blood clotting factors II, VII, and X in anticoagulant-resistant and susceptible Norway rats (*Rattus norvegicus* Berk.), *Comp. Biochem. Physiol. Part C Pharmacol. Toxicol. Endocrinol.* 122 (1999) 307–316. doi:10.1016/S0742-8413(98)10128-7.
- [38] I. Talstad, Which coagulation factors interfere with the one-stage prothrombin time?, *Haemostasis*. 23 (1993) 19–25. <http://www.ncbi.nlm.nih.gov/pubmed/8477905> (accessed July 18, 2016).

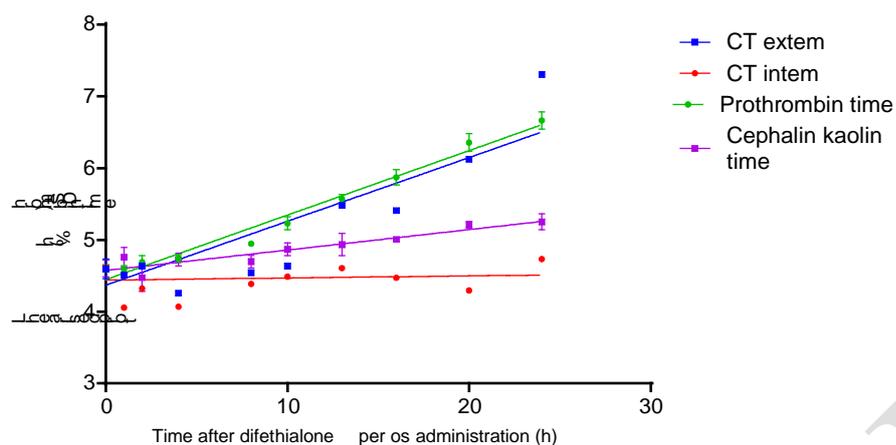
## 8 LEGENDS OF FIGURES

Fig 1



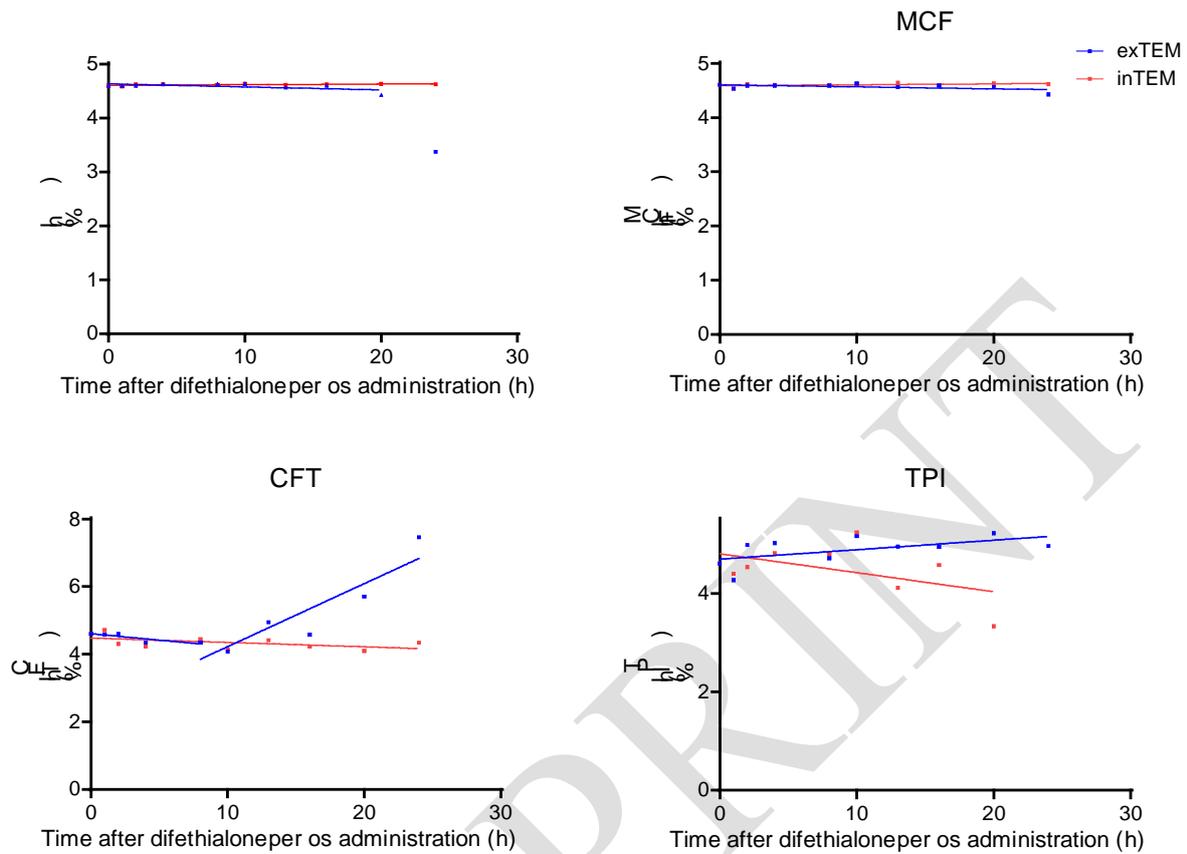
**Figure 1. Results given by different coagulation monitoring methods after *per os* administration of 10 mg.kg<sup>-1</sup> of difethialone. (A). clotting time tests. Values are presented for each time as the relative mean of clotting time and their standard deviation; for each kind of test, the basal mean of clotting times is set at 100%. (B) Vitamin K dependent clotting factors' activities. Values are presented for each time as the relative mean of activities and their standard deviation; for each factor, the basal activities' mean is set at 100%. (C) Silhouettes of the thromboelastometry graph with exTEM reagents. (D) Silhouettes of the thromboelastometry graph with inTEM reagents.**

Fig 2



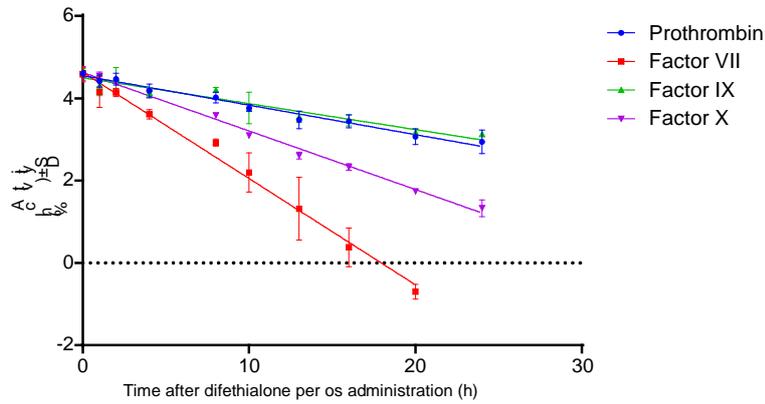
**Figure 2. Evolution of different clotting times after *per os* administration of 10 mg.kg<sup>-1</sup> of difethialone. For each parameter, values are expressed in percentage relative to the basal values' mean (values obtained at 0h), then they are linearized by a natural logarithmic function. Each point represent the mean of linearized values and its standard deviation.**

Fig 3



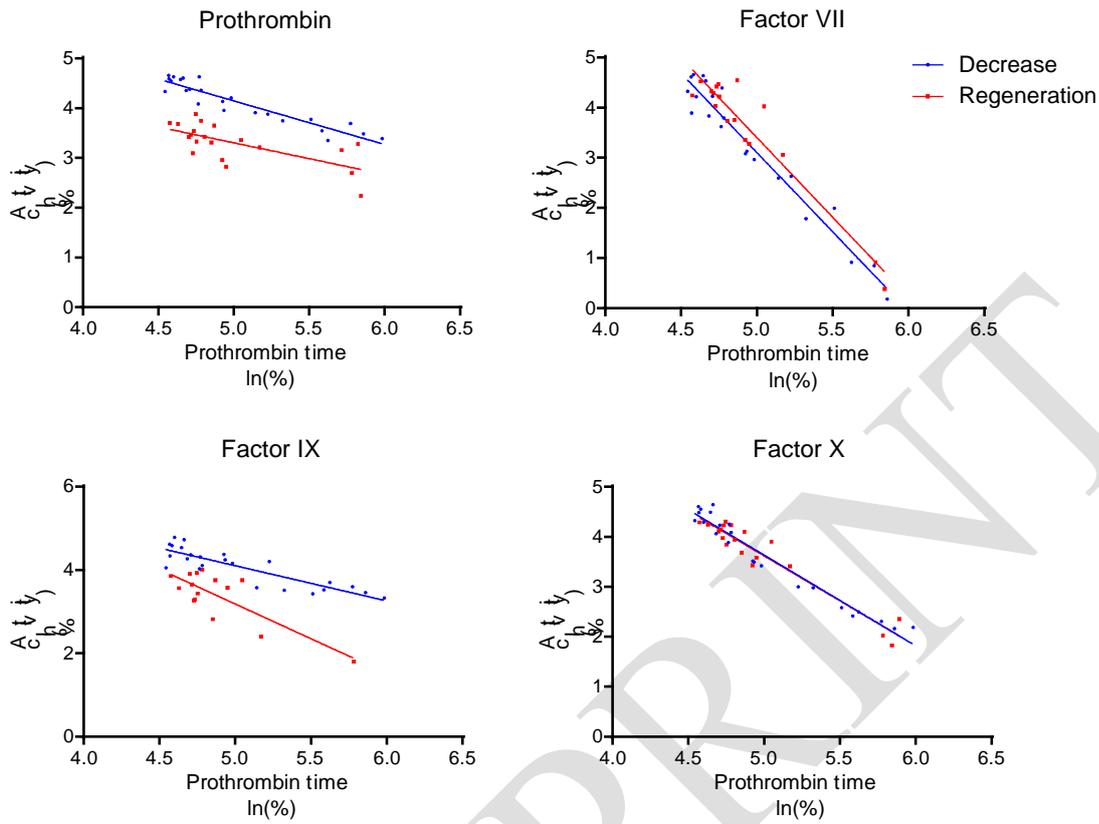
**Figure 3. Evolution of different TEM parameters after *per os* administration of 10 mg.kg<sup>-1</sup> of difethialone. For each parameter, values are expressed in percentage relative to the basal values' mean (values obtained at 0h), then they are linearized by a natural logarithmic function. Each point represent the mean of linearized values and its standard deviation.**

Fig 4



**Figure 4. Evolution of different clotting factors activity after *per os* administration of 10 mg.kg<sup>-1</sup> of difethialone. For each clotting factor, values are expressed in percentage relative to the basal values' mean (values obtained at 0h), then they are linearized by a natural logarithmic function. Each point represent the mean of linearized values and its standard deviation.**

Fig 5



**Figure 5. Evolution of clotting factors activity against prothrombin time during the decrease of coagulation capacity or its regeneration. For each couple “factor activity-prothrombin time”, values are expressed in percentage relative to the basal values’ mean of their respective parameters, then they are linearized by a natural logarithmic function.**

Fig 6

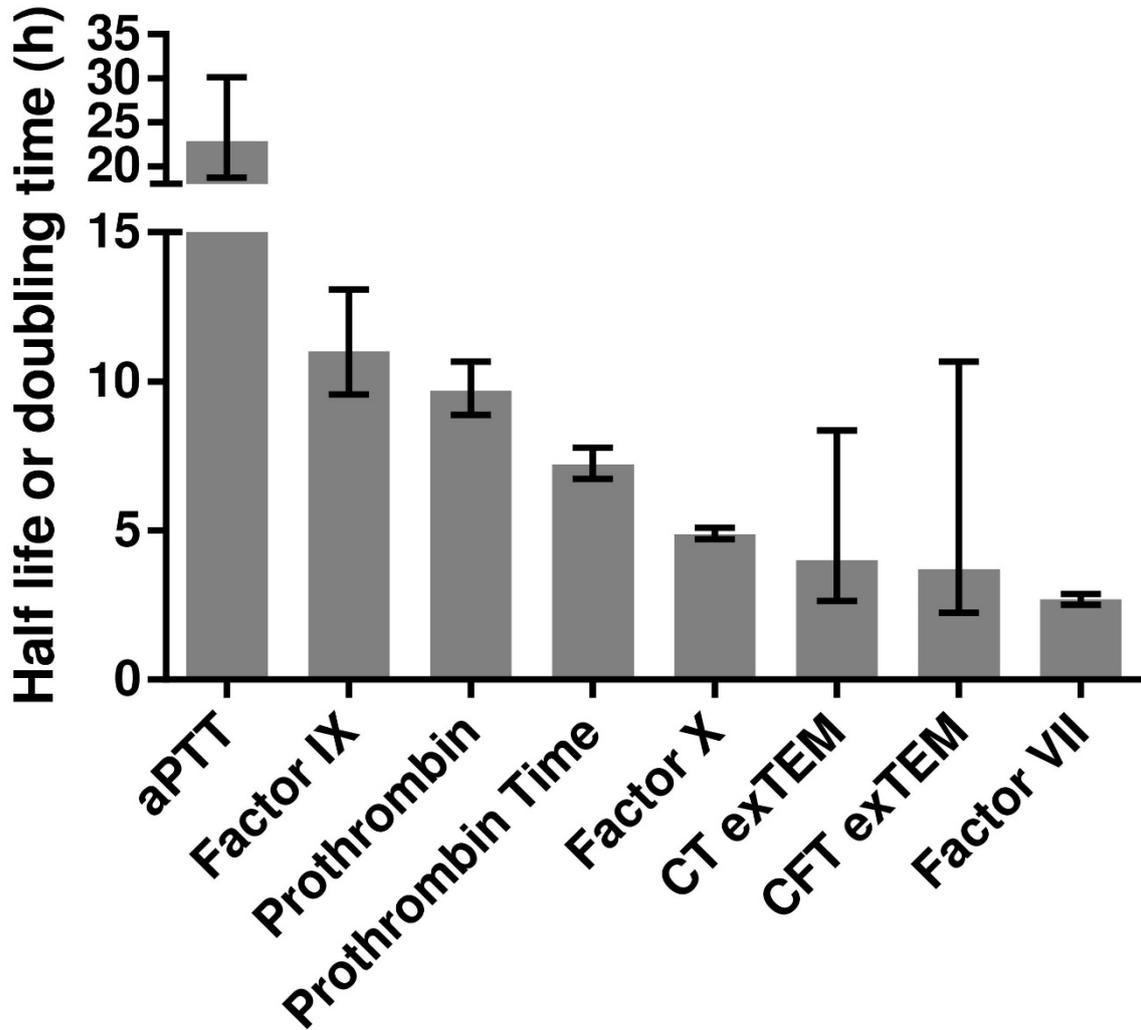


Figure 6. Values half-life or doubling time of each parameter evolution after per os administration of difethialone. Values are presented with their 95% confidence interval.

Parameters with no significant evolution are not represented.

PRE-PRINT

**Table 1. Values of parameters from linear regressions performed on linearized data of each clotting parameter evolution after *per os* administration of difethialone. Values are presented with their 95% confidence interval.**

Clotting parameters	Lag Time (h)	During the lag time		After the lag time		R Square	Doubling time
		Slope (h <sup>-1</sup> )	p-value	Slope (h <sup>-1</sup> )	p-value		
CT exTEM	10	-0.003 (-0.035 to 0.028)	0.819/*	0.173 (0.083 to 0.263)	0.0088	0.925	4.0 (2.6 to 8.4)
$\alpha$ exTEM	>20	-0.002 (-0.005 to 0.0002)	0.069	N/A	N/A	N/A	
MCF exTEM	>24	-0.002 (-0.004 to 0.0004)	0.098	N/A	N/A	N/A	
CFT exTEM	8	-0.038 (-0.079 to 0.003)	0.059	0.187 (0.065 to 0.308)	0.013	0.819	3.7 (2.3 to 10.7)
TPI exTEM	>24	0.008 (-0.001 to 0.018)	0.086	N/A	N/A	N/A	
CT inTEM	>24	0.003 (-0.013 to 0.018)	0.693	N/A	N/A	N/A	
$\alpha$ inTEM	>24	0.0004 (-0.00006 to 0.0009)	0.089	N/A	N/A	N/A	
MCF inTEM	>24	0.001 (-0.0003 to 0.002)	0.176	N/A	N/A	N/A	
CFT inTEM	>24	-0.005 (-0.013 to 0.001)	0.103	N/A	N/A	N/A	
TPI inTEM	>20	-0.017 (-0.042 to 0.008)	0.158	N/A	N/A	N/A	
Prothrombin time	2	0.044 (-0.022 to 0.111)	0.161	0.096 (0.089 to 0.103)	< 0.0001	0.972	7.2 (6.7 to 7.8)
aPTT	4	0.015 (-0.059 to 0.090)	0.661	0.030 (0.023 to 0.037)	< 0.0001	0.825	22.9 (18.7 to 30.1)

**Table 2. Values of parameters from linear regressions performed on linearized data of each clotting factor evolution after *per os* administration of difethialone. Values are presented with their 95% confidence interval.**

Clotting factor	Lag Time (h)	During the lag time		After the lag time			Half-life (h)
		Slope (h <sup>-1</sup> )	P-value	Slope (h <sup>-1</sup> )	p-value	R Square	
Prothrombin	0	NA	NA	-0.072 (-0.078 to -0.065)	<0.0001	0.937	9.7 (8.9 to 10.7)
Factor VII	0	NA	NA	-0.258 (-0.275 to -0.241)	<0.0001	0.972	2.7 (2.5 to 2.9)
Factor IX	2	-0.078 (-0.307 to 0.150)	0.441	-0.062 (-0.074 to -0.050)	<0.0001	0.843	11.0 (9.6 to 13.1)
Factor X	1	-0.049 (-0.274 to 0.177)	0.630	-0.144 (-0.150 to -0.137)	<0.0001	0.989	4.9 (4.7 to 5.1)

**Table 3. Summary of the upside and downside of each technique to monitor a VKA-dependent anticoagulation in rodents. Three characteristics, Precocity, reliability and Amplitude of evolution are respectively assessed according to lag time, R-square and absolute slope. A, applicable, Q, questionable, N, not applicable.**

Assay	Parameter	Applicability	Precocity	Reliability	Amplitude of evolution	
PT-Quick	Clotting time	A	+	++	+	
APTT	Clotting time	Q	+	-	-	
TEG/TEM-TF (ExTEM)	Clotting time	A	-	+	++	
	$\alpha$	N				
	MCF	N				
	CFT	Q	-	-	++	
	TPI	N				
TEG/TEM-CA (InTEM)	Clotting time	N				
	$\alpha$	N				
	MCF	N				
	CFT	N				
	TPI	N				
Specialty assays	Clotting factor activity	Factor II	A	+++	+	+
		Factor VII	A	+++	++	+++
		Factor IX	A	+	-	+
		Factor X	A	++	+++	++