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1 **Study of the efficiency of anticoagulant rodenticides to control *Mus***
2 ***musculus domesticus* introgressed with *Mus spretus Vkorc1***

4 *Running title: Resistance to rodenticides due to Vkorc1^{SPR} genotype in Mus musculus*
5 *domesticus*

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ABSTRACT

BACKGROUND : Antivitamin K anticoagulant (AVK) rodenticides are commonly used to control rodent pests worldwide. They specifically inhibit the VKORC1 enzyme essential for the recycling of vitamin K, and thus prevent blood clotting and cause death by haemorrhage. Numerous mutations or polymorphisms of the *Vkorc1* gene were reported in rodents and some lead to resistance to rodenticides. In house mice (*Mus musculus domesticus*), adaptative introgression of the *Vkorc1* gene from the Algerian mouse (*Mus spretus*) was reported. This adaptative introgression causes the substitution of four amino acids in *M. musculus domesticus*.

RESULTS: The consequences of introgression were assessed by (i) the characterization of the *in vivo* resistant phenotype of adaptative *Vkorc1*^{SPF}-introgressed mice, (ii) the characterization of the *ex vivo* resistance phenotype of the liver VKOR activity and (iii) the comparison of these results with the properties of recombinant VKORC1^{SPF} protein expressed in yeast. The resistance factor (from 1 to 120) induced by the four introgressed polymorphisms obtained using these three approaches was dependent on the used AVKs but were highly correlated among the three approaches.

CONCLUSION: The 4 introgressed polymorphisms were clearly the cause of the strong resistant phenotype observed in the field. In the context of strong selection pressure due to the extensive use of AVK, this resistant phenotype may explain the widespread distribution of this genotype from Spain to Germany.

Keywords: *Mus musculus domesticus* ; *Mus spretus* ; antivitamin K ; VKORC1 ; resistance ; mutation

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1 INTRODUCTION

Rodents are responsible for the degradation and the destruction of numerous cereal crops. They are carriers of many zoonotic pathogens. Moreover, they are responsible for damages to substructures, electronic or electric networks to infrastructure. Antivitamin K molecules (AVKs) are the main chemical rodenticides used in the world. These molecules block the vitamin K recycling and thus limit the production of the vitamin K-dependent clotting factors (II, VII, IX and X)¹. As a consequence, AVKs provoke an internal hemorrhage and the death of the intoxicated rodents. The VKOR enzyme is encoded by the *Vkorc1* gene simultaneously described in 2004 by Li et al² and Rost et al³. The extensive use of these rodenticides for more than 50 years led to the selection of rodents resistant to certain compounds of this class. Two resistant mechanisms were described. A metabolic resistance due to the increased metabolism of AVKs as suggested in roof rats (*Rattus rattus*)^{4,5} which could be also detected in brown rats (*Rattus norvegicus*) even in association with the target resistance mechanism^{6,7}. The target resistance corresponds to mutations or polymorphisms (SNPs) in the *Vkorc1* gene resulting in a decrease of the inhibitory effect of AVKs towards the VKOR activity catalyzed by VKORC1 enzyme. Numerous mutations or SNPs have been detected in this gene that are associated with resistant phenotype in rats⁸⁻¹⁰, mice^{8,9} and humans^{11,12}. The use of recombinant VKORC1 enzymes expressed in yeast^{10,12,13} appears to be a useful tool to study the resistance associated with *Vkorc1* mutations or SNPs. This experimental approach was recently challenged by other indirect approaches designed as cell-based VKOR assays^{14,15}. Nevertheless, the resistance factors conferred by p.Tyr139Phe, p.Tyr139Cys and p.Tyr139Ser SNPs determined using the

1 recombinant enzymes were positively correlated with the results obtained by *ex vivo* and *in vivo* assays^{16,17}.

2 In house mice (*Mus musculus domesticus*) three SNPs seem to be particularly frequent, the
3 p.Leu128Ser and p.Tyr139Cys SNPs and a set of 3 or 4 SNPs p.Arg12Trp, p.Ala26Ser,
4 p.Ala48Thr and/or p.Arg61Leu. This set of 3 or 4 SNPs seems to be the consequence of the
5 adaptive introgression of *Vkorc1* gene from the Algerian mouse (*Mus spretus*) into the
6 genome of *Mus musculus domesticus*¹⁸. A part of about 20 MB around the *Vkorc1* gene in
7 chromosome 7 of *Mus spretus* was introgressed in the *Mus musculus domesticus* genome¹⁹.
8 Other pieces of the *Mus spretus* genome were also introgressed in the *Mus musculus*
9 *domesticus* genome but the consequences are unknown. An initial crossing between these two
10 mice species might have occurred in their cohabitation area (North Africa or Spain).
11 Descendants that mated with *Mus musculus* acquired the *Vkorc1* gene of the *Mus spretus*
12 species (*Vkorc1*^{SPR}). *Mus musculus* with *Vkorc1*^{SPR} were found in Spain and Germany, but not
13 in Italy or Greece and appear to be resistant to coumatetralyl, bromadiolone and
14 difenacoum¹⁸.

15 In France, such mice were trapped in a French bakery of Lyon and were considered resistant
16 to AVKs by pest control operators. After establishing a strain with these mice in our animal
17 facilities, we characterized their *in vivo* resistant phenotype, the *ex vivo* resistance phenotype
18 of their liver VKOR activity and compared these results with the resistance factors obtained
19 using the recombinant VKORC1^{SPR} protein expressed in yeast.

2 MATERIALS AND METHODS

2.1 Animals

20 Twenty *Mus musculus domesticus* wild mice resistant to rodenticides were trapped in a
21 French bakery of Lyon (France). Mice were acclimated in the animal facilities of VetAgro

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3 1 Sup in Lyon (France) and maintained by intercrossing. Animals received a standard feed
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5 2 (Scientific Animal Food and Engineering, reference A04 for adult rodent, Augy, France) and
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7 3 water *ad libitum*. Experimental research on these mice was performed according to an
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9 4 experimental protocol following international guidelines and approved by the ethics
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11 5 committee of the Veterinary School of Lyon.
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16 7 **2.2 Species identification**
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18 8 Species identification was based on mitochondrial *cytochrome b* sequence and conducted as
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20 9 previously described by Goulois et al¹³.
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25 11 **2.3 *Vkorc1* sequencing**
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27 12 In order to sequence the totality of the *Vkorc1* gene, two sets of primers were used. The
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29 13 sequences of the first set of primers sVKOR-S1 and sVKOR-AS1 were (5'-
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31 14 GATTCTTCCTCCTGTCC-3') and (5'-AGACCCTGTCTCAAAACCTA-3') and were
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33 15 used to amplify mouse *Vkorc1* gene from nucleotide -36 to nucleotide +1727. The sequences
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35 16 of the second set of primers sVKORC1-S2 and sVKORC1-AS2 were (5'-
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37 17 GAAAGCAGAACACTTAGCAGG-3') and (5'-AACCAACAGCAGAATGCAGCC-3') and
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39 18 were used to amplify the mouse *Vkorc1* gene from nucleotide (+)1252 to nucleotide (+)2512.
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41 19 Mouse *Vkorc1* amplifications were performed using sVKOR-S1 and sVKOR-AS1 or
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43 20 sVKOR-S2 and sVKOR-AS2 (10 pmol), Accuprime polymerase (1 units, Invitrogen) in a 25
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45 21 µl reaction volume containing 2 µl DNA, 2.5 µl 10X Accuprime buffer and 200 µM of each
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47 22 deoxynucleotide triphosphate. The amplification was performed at 94°C for 3 min followed
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49 23 by 40 cycles at 94°C for 20 s, 64°C for 20 s, 68°C for 50 s, and a final extension step at 68°C
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51 24 for 10 min. The amplified products were sequenced on both strands; the obtained sequences
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were compared to published *Mus musculus domesticus* (Genbank accession n°AC124602) and *Mus spretus Vkorc1* sequences (Genbank accession n°GQ905711).

2.4 Genotyping

This genotyping method was used to specifically detect the R61W mutation. Genomic DNA was extracted from a tissue sample (tail) using the Macherey-Nagel Nucleo-spin Tissue extraction kit (Hoerd, France). The allele-specific PCR method employed two pairs of primers purchased from Eurogentec (Angers, France). The forward primer was one of the two primers which the three prime nucleotide of the first one matched the *Vkorc1* sequence of the SD strain (i.e. the rat genome sequence of the *Vkorc1*; R61-primer: 5'TCTCCTCTCGGTGGGGCCT^{3'}) and the three prime nucleotide of the other matched the mutation in the *Vkorc1* sequence of the donor strain (L61-primer: 5'TCTCCTCTCGGTGGGGCCG^{3'}). The reverse primers matched the *Vkorc1* sequence of the rat genome sequence for the *Vkorc1* (5'CAGGCAACCAAATATGCTGT^{3'}). We amplified 106 base pairs of *Vkorc1* gene using quantitative real-time PCR (qPCR) in a Thermocycler Mx3000P Stratagene (Massy, France). The reaction mixture contained 5X HOT BIOAmp EvaGreen qPCR Mix (no ROX). The 15 µL reactions contained 2µL of genomic DNA, 1pmol/µL of each forward and reverse primers, 5x EvaGreen qPCR buffer including dNTPs, HOT BIOAmp DNA polymerase, MgCl₂ at 12.5mM, EvaGreen dye. The cycling was initiated by one denaturation step at 95°C for 15 min; followed by 40 cycles of 95°C for 30 s, 65°C for 40 s and 72°C for 30 s each. Fluorescence from both SYBER dyes was recorded at the annealing step. The point at which the PCR product is first detected above a fixed threshold, the thermal cycle threshold (C_t), was determined for each sample in duplicate, and the average C_t of duplicate sample was calculated. The three possible genotypes R61/R61, R61/L61 and L61/L61 were deduced from the differences between their characteristic cycle

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1 threshold values (ΔCt), that is the difference of Ct values between the matched and the
2 mismatched primer extension for homozygous mice, and the absence of such a difference in
3 Ct values for heterozygous animals.

5 **2.5 No-choice feeding test with AVK**

6 No-choice feeding tests were performed on mice previously genotyped as homozygous for
7 *VkorcI^{spr}* genotype or *VkorcI^{dom}* genotype. Mice were divided according to their genotype in
8 groups of 10 male or female mice. Individuals were weighed before the start of the test. Mice
9 were fed with commercial ready baits containing either 0.0025% difethialone, 0.005%
10 brodifacoum, 0.005% difenacoum, 0.005% flocoumafen, 0.005% chlorophacinone, 0.005%
11 bromadiolone, and 0.0375% coumatetralyl. Feeding periods of 4 days were used for males
12 and females homozygous for *VkorcI^{spr}* or *VkorcI^{dom}* genotype. Remaining bait was weighed
13 daily and the intake of bait recorded. Mortality of mice was also observed daily. At the end of
14 the feeding period with toxic baits, animals were given diet A04 again, water *ad libitum* and
15 observed daily for 17 days. The criterion for resistance was survival for 21 days.

17 **2.6 AVK concentration in liver**

18 Residues of AVK were measured in liver when mice homozygous for *VkorcI^{spr}* and
19 *VkorcI^{dom}* were found dead during the no-choice feeding test in order to compare residue
20 concentration between genotypes. Liver tissue (1g) was extracted with acetone by using an
21 Ultra Turrax tissue disperser from IKA Labortechnik ® (VWR International, Strasbourg,
22 France). Extract was centrifuged at 3000 rpm for 10 min. Supernatant was thus evaporated at
23 60°C under a gentle nitrogen flow. Dry extract was resuspended in acetonitrile/hexane
24 (50%/50%). The hexane layer was eliminated and the rest was dried at 60°C under gentle
25 nitrogen flow. The final dry extract was dissolved in methanol and AVK concentrations were

analysed by HPLC on a reverse phase C-18 column (4.6×150 mm, 5 μ m; Waters, Milford, MA, U.S.A.) at a detection wavelength of 258, 260 and 267 nm for difenacoum, difethialone and brodifacoum, respectively. The C-18 column was heated to 40°C for difenacoum and brodifacoum and 30°C for difethialone. A gradient elution system was used with a flow rate of 1 mL/min: from 30% methanol/70% water (acidified with 0.2% H₃PO₄) to 70% methanol/30% water at 10 min for difenacoum; from 30% acetonitrile/70% water (acidified with 0.1% H₃PO₄) to 90 acetonitrile/10% water at 14 min for brodifacoum and from 60% acetonitrile/40% water (acidified with 0.2% H₃PO₄) to 75 % acetonitrile/25% water at 10 min for difethialone.

2.7 Preparation of liver microsomes

Liver cell microsomes were prepared from fresh liver cells by differential centrifugation, as described previously by Moroni et al²⁰. Protein concentrations were evaluated by the method of Bradford²¹ using bovin serum albumin as a standard. Microsomes were frozen at -80°C and used for kinetic analysis.

2.8 Heterologous expression in *Pichia pastoris*

Heterologous expressions of *Mus musculus domesticus* and *Mus spretus* VKORC1 proteins were performed in *Pichia pastoris* as described previously¹⁰.

2.9 Vitamin K epoxide reductase activity (VKOR) assays and kinetics

Microsomal VKOR activity was assayed as described previously²² and characterization of the VKOR activity was performed by the determination of K_m , V_{max} and K_i towards various AVKs as described previously^{10,12,13,22}. Resistance factor of the VKOR activity catalyzed by

1 VKORC1^{spr} was calculated by dividing the K_i obtained for VKORC1^{spr} by the corresponding
2 K_i obtained for VKORC1^{dom}.

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14 **2.10 Statistical analysis**

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16 In no-choice feeding tests, comparison of survival between treatments was done using a Log-
17 rank test. Comparison of total consumption of active substance between groups was done
18 using Kruskal-Wallis tests with the R-fit program.

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20 For enzymatic analysis, data are presented as the mean $\pm$ SD of three individual
21 measurements. Multiple comparisons between groups were done using Kruskal-Wallis tests.
22 Comparison between two groups was done using Mann-Whitney tests.

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32 **3 RESULTS**

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34 **3.1 Description of founder population**

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36 Amplified *cytochrome b* sequences of trapped mice had 99% of homology with sequences of
37 *cytochrome b* published for *Mus musculus domesticus*. Moreover, four nucleotide changes
38 were identified in the *Vkorc1* gene of trapped mice compared to the published *M. musculus*
39 *domesticus Vkorc1* sequence: the g.34C>T, g.76G>T, g.111A>G, g.142G<A nucleotide
40 changes in exon 1 and the g.976G>T nucleotide change in exon 2 of the *Vkorc1* gene,
41 genotype corresponding to *Vkorc1*^{spr} genotype. These nucleotide changes led to p.Arg12Trp,
42 p.Ala26Ser, p.Glu37Glu, p.Ala48Thr and p.Arg61Leu SNPs.
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54 **3.2 In vivo resistance due to *Vkorc1*^{spr} genotype versus anticoagulant molecules**
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Mortality results and mean anticoagulant molecule consumption obtained for mice homozygous for *VkorcI^{spr}* genotype are presented in Table 1. At day 21, 100% of the female mice homozygous for *VkorcI^{spr}* genotype treated with coumatetralyl (375 ppm), chlorophacinone (50 ppm) and bromadiolone (50 ppm) were alive despite normal values of consumption of baits, while 80% of the female mice treated with difenacoum (50 ppm) and 100% treated with difethialone (25 ppm), brodifacoum (50 ppm) and flocoumafen (50 ppm) were dead. For males, 80, 50 and 40% of the mice treated, respectively, with coumatetralyl (375 ppm), chlorophacinone (50 ppm) or bromadiolone (50 ppm) were still alive despite normal consumption of baits, while 100% of the male treated with difenacoum (50 ppm), difethialone (25 ppm), brodifacoum (50 ppm) or flocoumafen (50 ppm) were dead. For mice homozygous for *VkorcI^{dom}*, mortality was higher than 90% for both sexes whatever the used AVK was, while consumption of baits was similar to that measured for mice homozygous for *VkorcI^{spr}* genotype (data not shown). Concentrations of residues of AVK in livers at the death of animals were similar between genotype, except for males that died in trials with difenacoum where AVK concentration was 5 fold-higher in mice homozygous for *VkorcI^{spr}* compared to mice homozygous for *VkorcI^{dom}* (Table 2).

3.3 Characterization of the VKOR activity catalyzed by liver microsomes of mice homozygous for *VkorcI^{spr}* genotype

Kinetics parameters of the VKOR activity catalyzed by liver microsomes of male or female mice homozygous for *VkorcI^{spr}* genotype or homozygous for *VkorcI^{dom}* genotype were analyzed by measuring the velocity of the reaction as a function of the vitamin K epoxide concentration (Table 3). In male and female mice homozygous for *VkorcI^{spr}* genotype, apparent $V_{\max}$ were 33.4 ± 4.0 and 53.6 ± 8.1 pmol.min⁻¹.mg⁻¹; apparent K_m were 22.8 ± 9.6 and 34.9 ± 15 .

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Independent of AVKs used, apparent K_m was never modified by the addition of anticoagulant molecules while apparent V_{max} was lowered by the use of anticoagulant molecules. Modeling of the data using a non-competitive equation gave a correct fit allowing the determination of K_i parameters. Systematically, K_i constants were higher in mice homozygous for $VkorcI^{spr}$ genotype compared to mice homozygous or heterozygous for $VkorcI^{dom}$ genotype (Table 4).

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3.4 Characterization of the VKOR activity catalyzed by yeast microsomes expressing *Mus spretus* VKORC1 proteins and *Mus musculus* VKORC1 proteins.

The VKOR activity catalyzed by recombinant yeast expressing either VKORC1^{dom} or VKORC1^{spr} was a linear function of time and protein (results not shown). The reaction rates of recombinant VKORC1 enzymes towards vitamin K₁ epoxide followed the Michaelis–Menten model allowing the determination of kinetic parameters. K_m were 25 μ M when the reaction was catalyzed by recombinant yeast expressing VKORC1^{dom} ; 24 μ M when the reaction was catalyzed by recombinant yeast expressing VKORC1^{spr}.

All AVKs were able to inhibit the VKOR activity catalyzed by VKORC1^{dom} and VKORC1^{spr} proteins, but the concentrations use to inhibit VKORC1^{spr} were much stronger than those required to inhibit VKORC1^{dom}. Apparent K_m was not modified by the addition of AVKs, while apparent V_{max} was lowered. Modeling of the data using non-competitive equation gave a correct fit allowing the determination of K_i parameters for VKORC1^{dom} and VKORC1^{spr}, and thus calculation of resistance factors induced by the four *Vkorc1* SNPs for each AVK (Fig. 1). Depending on the AVK, the resistance factor varied from 2.3 for difethialone to 35 for chlorophacinone.

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4 DISCUSSION

House mice with the four SNPs p.Arg12Trp, p.Ala26Ser, p.Ala48Thr and p.Arg61Leu corresponding to the *VkorcI*^{SPR} genotype are present in France (specifically in Lyon). The sequence of the cytochrome b of these mice has high homology (99%) with the sequence of *Mus musculus domesticus* and more limited homology (92%) with *Mus spretus*. This specific genotype stems from an introgression on chromosome 7 of the house mouse by hybridization of the house mouse with the Algerian mouse (*Mus spretus*)¹⁹. This genotype was described for the first time in house mice trapped in Westphalia in Germany^{18,23}. Since, then *VkorcI*^{SPR} introgressed house mice were detected in diverse geographical areas of Germany, Switzerland^{9,18} and Spain¹⁸. In Spain, 93% of the house mice were reported to be carriers for *VkorcI*^{SPR} genotype¹⁸. In England, Italy and Greece, no *VkorcI*^{SPR} introgressed house mouse were detected. In France, the presence of such mice was previously unknown. Nevertheless, France is located between Germany and Spain where *VkorcI*^{SPR} introgressed mice were found. Moreover, in southern France, the co-existence of *Mus musculus domesticus* with *Mus spretus* is described²⁴. It is thus not surprising that *VkorcI*^{SPR} introgressed house mice are present in France. These mice were captured in a French bakery invaded by mice despite the use of different anticoagulant rodenticides in the last 6 months (*i.e.*, baits with 50 ppm of bromadiolone or 50 ppm of brodifacoum). Resistance to rodenticides of this population of mice was suspected and *in vivo* tested by no-choice feeding trials. Despite consumption of coumatetralyl or chlorophacinone 8 to 10-fold higher than their LD50, no mortality of *VkorcI*^{SPR} introgressed female mice was observed in our study and only 20% and 50% of male mice died after consumption of 6-fold the LD50 of coumatetralyl or chlorophacinone. This resistance to coumatetralyl is in agreement with the results reported for a population of *VkorcI*^{SPR} introgressed mice trapped in Germany¹⁸. The first generation AVKs such as coumatetralyl and chlorophacinone can be thus considered as inefficient to control the populations of *VkorcI*^{SPR} introgressed mice, even if males seem to be less resistant than

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females. Bromadiolone and difenacoum, belonging to second generation molecules, seem also ineffective for controlling the populations of *VkorcI^{spr}* introgressed mice, particularly to control females. No female was found dead after the consumption of 10-fold the LD50 of bromadiolone, as described for the *VkorcI^{spr}* introgressed mice trapped in Germany¹⁸ and only 80% of females were found dead after consumption of 5.5 mg.kg⁻¹ of difenacoum for 4 days. The other second generation molecules (*i.e.*, difethialone, brodifacoum and flocoumafen) were efficient to control *VkorcI^{spr}* introgressed mice. Consumption of only 4-fold the LD50 of difethialone (*i.e.*, 1.9 mg.kg⁻¹.day⁻¹ for 4 days) leads to 100% mortality. For brodifacoum and flocoumafen, 100% mortality was obtained with highest concentrations (*i.e.*, 2.8 and 7.8 mg.kg⁻¹.day⁻¹ for 4 days) of active substance consumed. However, low concentrations of brodifacoum or flocoumafen were not tested because commercial available baits containing less than 50 ppm of active substance were not available.

To further the understanding of resistance, we compared the observed phenotype with the properties of liver microsomal VKOR activity. The *K_i* towards first generation molecules and bromadiolone for liver microsomes of *VkorcI^{spr}* introgressed mice were almost 10- to 100-fold higher than those obtained for liver microsomes of *VkorcI^{dom}* mice (Fig. 1), while *K_i* towards second generation molecules such as difethialone, brodifacoum and flocoumafen were very similar between genotypes. These results are coherent with the *in vivo* phenotype of the *VkorcI^{spr}* introgressed mice. Such a demonstration of the coherence between the properties of the VKOR activity and the *in vivo* resistance phenotype to AVKs was first performed by Lasseur et al¹⁷ and Grandemange¹⁶ et al for the Y139F mutation detected in *VkorcI* of *Rattus norvegicus*.

To demonstrate the involvement of the four introgressed SNPs in the VKOR activity measured in the liver of *VkorcI^{spr}*-introgressed mice, we produced yeast expressing either *VkorcI^{dom}* or *VkorcI^{spr}* and determined resistance factor. This resistance factor corresponded

1 to the ratio of K_i obtained for yeast microsomes expressing $VkorcI^{spr}$ with K_i obtained for
2 yeast microsomes expressing $VkorcI^{dom}$. Clearly, the four SNPs modified the resistance to
3 first generation AVKs of the VKORC1 enzyme and these modifications are similar to the
4 modifications obtained for liver microsomes. The use of recombinant VKORC1 enzymes
5 gave direct information about the catalytic properties of VKOR activity of the liver which is
6 clearly linked to the *in vivo* resistance phenotype. This study is a supplementary
7 demonstration revealing that the *in vitro* characterization of microsomal VKOR activity in
8 yeast or in liver by the DTT-driven VKOR assay is perfectly reflected the *in vivo* phenotype
9 when no other mutation than the mutation or SNP in *VkorcI* gene is involved in the
10 resistance.

11 Indeed, additional genetically based mechanisms could contribute to the physiological
12 response to AVK exposure and the presence of numerous fragments of the *Mus spretus*
13 genome in the introgressed *Mus musculus* genome¹⁹ could be associated to resistance. Altered
14 expression of several cytochromes^{4,5,7} was proposed to mediate or be involved in resistance to
15 AVK. The concentrations of AVK molecules in the liver of $VkorcI^{spr}$ introgressed mice and
16 $VkorcI^{dom}$ mice were similar, suggesting no difference between expressions of cytochrome
17 genes between genotypes. All these observations taken together are in favor of the exclusive
18 involvement of the $VkorcI^{spr}$ genotype in the *in vivo* resistance phenotype observed.
19 Nevertheless, in the absence of a 3D-structure of VKORC1 enzymes of *Mus musculus*
20 *domesticus* and *Mus spretus*, it is difficult to understand the structural consequences of the
21 four introgressed SNPs and thus the consequences of these SNPs on the binding of first
22 generation, while binding of second generation AVKs does not seem to be modified by these
23 SNPs.

24 The characterization of VKOR activity allowed also determining the consequences of the four
25 introgressed SNPs for the efficiency of the enzyme to reduce vitamin K epoxide in vitamin K

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1 quinone. These SNPs lead to an enzyme with an altered enzymatic efficiency (i.e., V_{max}/K_m)
2 compared to *VkorcI*^{dom} mice, thus reduce the efficiency of the vitamin cycle and consequently
3 increase the vitamin K intake requirements for mice homozygous for *VkorcI*^{spr} genotype. This
4 decreased enzymatic efficiency is not in favor of an adaptation of these mice to a granivorous
5 vitamin K-deficient diet¹⁸. On the other hand, *Mus spretus* could be exposed to plants
6 containing natural coumarin derivatives such as ferulenol contained in *Ferula communis*
7 found exclusively in the Mediterranean basin^{25,26} and the molecular evolution of *VkorcI* in
8 *Mus spretus* could result from this exposure.
9 The results presented demonstrate the necessity of using the most powerful second generation
10 molecules such as difethialone, brodifacoum or flocoumafen to control populations of
11 *VkorcI*^{spr} introgressed mice. The use of first generation molecules on a site where *VkorcI*^{spr}
12 introgressed mice are present even with low frequency would promote the build-up of the
13 *VkorcI*^{spr} allele in this population, thereby increasing the frequency of homozygous animals
14 from initially rare to ultimately very common. Nevertheless, the first generation molecules are
15 environmentally safe and should be favored when absence of resistance is demonstrated. This
16 demonstration could be made by preliminary genotyping. In this case, evaluation of resistance
17 induced by single or combined mutation(s) to anticoagulant molecules seems to be crucial. In
18 mice, a large number of SNPs was already identified p.Glu37Gly, p.Arg58Gly, p.Trp59Gly,
19 P.Trp59Leu, p.Trp59Ser, p.Leu124Gln, p.Leu128Ser, p.Tyr139Cys^{8,9,17,23}, but resistance
20 associated to these SNPs has not been fully characterized, except now for *VkorcI*^{spr} genotype.
21 Detection of SNPs or mutations on the *VkorcI* gene does not always mean resistance to AVK.
22 Indeed, in humans, more than 26 missense mutations of the *VKORC1* gene have been found,
23 but only 6 mutations lead to severe resistance to AVK¹⁰. Therefore, consequences of *VkorcI*
24 SNPs or mutations detected in rodents, and especially in mice, should be characterized in
25 order to use the most appropriate AVK.

5 CONCLUSION

The present study demonstrates the existence of *Vkorc1*^{SPF} genotype introgressed house mice in France. The *in vivo* characterization performed in this study will allow in the future targeting the appropriate molecules when this specific genotype is detected. Indeed, mice are totally resistant to the first generation AVK and only the use of the most powerful second generation AVK will control populations. This resistance phenotype was shown in this study to be exclusively due to the presence of the four introgressed SNPs p.R12W, p.A26S, p.A48T and p.R61L in *Vkorc1*.

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LEGENDS OF FIGURES

Figure 1. Inhibition effect of various anticoagulants on VKOR activity catalyzed by yeast microsomes expressing VKORC1^{spr} or by liver microsomes of *Vkorc1*^{spr} genotype introgressed house mice, compared to the effect measured in yeast microsomes expressing VKORC1^{dom} or by liver microsomes of mice carriers for wild type *Vkorc1*.

MALES					FEMALES		
Bait containing :	LD50 (mg/kg)	Total consumption of active substance (µg/mouse for 4 days)	Mortality (%)	Mortality interval (days)	Total consumption of active substance (µg/mouse for 4 days)	Mortality (%)	Mortality interval (days)
Coumatetralyl	15-30	3240 (27 mg/kg/day)	20	4 to 7	3559 (31.8 mg/kg/day)	0	-
Chlorophacinone	3.15	574 (4.8 mg/kg/day)	50	3 to 4	844 (7.5 mg/kg/day)	0	-
Bromadiolone	1.75	639 (5.3 mg/kg/day)	60	4 to 7	501 (4.5 mg/kg/day)	0	-
Difenacoum	0.8	453 (3.8 mg/kg/day)	100	4 to 7	618 (5.5 mg/kg/day)	80	7 to 14
Difethialone	1.9	237 (1.9 mg/kg/day)	100	4 to 7	294 (2.6 mg/kg/day)	100	7 to 14
Brodifacoum	0.4	333 (2.8 mg/kg/day)	100	4 to 9	471 (4.2 mg/kg/day)	100	7 to 11
Flocoumafen	0.54	930 (7.8 mg/kg/day)	100	4 to 10	904 (8.1 mg/kg/day)	100	7 to 14

Table 1: Consumption of active substance and mortality of house mice homozygous for *VkorcI*^{SPR} genotype during a no-choice feeding test with various AVKs.

Bait containing :	[AVK] in liver of mice with $Vkorc1^{dom}$	
	[AVK] in liver of mice with $Vkorc1^{spr}$	
	Male	Female
Difenacoum (50 ppm)	0.21	0.96
Difethialone (25 ppm)	0.88	1.06
Brodifacoum (50 ppm)	1.31	1.00

Table 2: Ratio of AVK residues in liver of house mice homozygous for $Vkorc1^{dom}$ and house mice homozygous for $Vkorc1^{spr}$, that died during no-choice feeding tests.

		K_m (μM)	V_{max} ($pmol.min^{-1}.mg^{-1}$)	V_{max}/K_m ($\mu L.min^{-1}.mg^{-1}$)
Male	$VkorcI^{spr}/VkorcI^{spr}$	22.8 ± 9.6	33.4 ± 4.0	1.46
	$VkorcI^{dom}/VkorcI^{dom}$	13.6 ± 2.0	54.8 ± 1.0	4.02
Female	$VkorcI^{spr}/VkorcI^{spr}$	34.9 ± 15	$53.6 \pm 8.1^*$	1.14
	$VkorcI^{dom}/VkorcI^{dom}$	16.8 ± 3.8	151 ± 9.3	8.99

Table 3. Apparent kinetic parameters towards vitamin K1 epoxide for VKOR activity in liver of house mice homozygous for $VkorcI^{spr}$ genotype or homozygous for $VkorcI^{dom}$ genotype. * $p<0.05$ compared to the corresponding data obtained for liver of female mice homozygous for $VkorcI^{dom}$ genotype.

	MALES		FEMALES	
	<i>VkorcI^{spr}/VkorcI^{spr}</i>	<i>VkorcI^{dom}/VkorcI^{dom}</i>	<i>VkorcI^{spr}/VkorcI^{spr}</i>	<i>VkorcI^{dom}/VkorcI^{dom}</i>
	(nM)	(nM)	(nM)	(nM)
Coumatetralyl	662 ± 31	35 ± 2.5 ^{**}	955 ± 487	38 ± 4 [*]
Chlorophacinone	354 ± 28	26 ± 1.5 ^{**}	572 ± 159	44 ± 1 [*]
Bromadiolone	247 ± 17	36 ± 3.1 ^{**}	387 ± 150	28 ± 4
Difenacoum	92 ± 32	17 ± 2.1	96 ± 35	30 ± 3
Difethialone	70 ± 8	21 ± 2.9 [*]	32 ± 11	50 ± 7
Brodifacoum	76 ± 16	25 ± 3.3 [*]	51 ± 23	38 ± 6
Flocoumafen	28 ± 5	7.2 ± 1.2 [*]	13 ± 4	10 ± 2.1

Table 4: K_i values towards various AVK obtained with liver microsomes of house mice homozygous for *VkorcI^{spr}* genotype or homozygous for *VKorcI^{dom}* genotype. ^{**} $p < 0.01$ or ^{*} $p < 0.05$ compared to the corresponding data obtained for liver of male mice homozygous for *VkorcI^{dom}* genotype. K_i were found to be statistically different between molecules ($p < 0.01$) within *VkorcI^{spr}* genotype or *VkorcI^{dom}* genotype.

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Figure 1

Figure 1 : Inhibition effect of various anticoagulants on VKOR activity catalysed by yeast microsomes expressing VKORC1spr or by liver microsomes of Vkorc1spr genotype introgressed house mice, compared to the effect measured in yeast microsomes expressing VKORC1dom or by liver microsomes of mice carriers for wild type Vkorc1.

338x190mm (96 x 96 DPI)