SCIENTIFIC OPINION

Genetic TSE resistance in goats[1]

Scientific Opinion of the Panel on Biological Hazards

(Question No EFSA-Q-2008-774)

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PANEL MEMBERS

Olivier Andreoletti, Herbert Budka, Sava Buncic, Pierre Colin, John D. Collins, Aline De Koeijer, John Griffin, Arie Havelaar, James Hope, Günter Klein, Hilde Kruse, Simone Magnino, Antonio Martinez López, James McLauchlin, Christophe Nguyen-Thé, Karsten Noeckler, Birgit Noerrung, Miguel Prieto Maradona, Terence Roberts, Ivar Vågsholm, Emmanuel Vanopdenbosch.

SUMMARY

Following a request from the European Commission, the Scientific Panel on Biological Hazards (BIOHAZ) was asked to deliver a scientific opinion on genetic TSE resistance in goats in consideration of a pilot project study carried out in Cyprus. In particular, the BIOHAZ Panel was requested to assess the scientific validity of the study and to indicate to what extent and based on this study genetic breeding can be used as a control program for Classical scrapie in goats in Cyprus.

The review of the limited studies carried out so far indicate that *PRNP* polymorphisms can modulate scrapie susceptibility in goats. Some polymorphisms could be associated with a lower susceptibility to TSE and are possible candidates for future breeding programmes. It has to be clarified whether the association of *PRNP* polymorphisms with apparent resistance to scrapie in goat is limited to those scrapie agents involved in the outbreaks studied and if this apparent resistance can be extended to other combinations of TSE agents/goat breeds.

The report of the Cypriot pilot study presents a case control study in Cypriot goat herds aiming at the identification of the effect of *PRNP* polymorphisms on TSE susceptibility. According to the author, the results obtained would indicate a higher resistance against clinical disease in goats bearing the H154, D146 or S146 *PRNP* polymorphism than in goats bearing R154 or N146.

A re-analysis of the raw data of the Cypriot study carried out was in broad agreement with the initial analysis presented in the report. However, this can not be extended to infer an association between H154, D146 and S146 *PRNP* alleles and resistance to infection nor could

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it be extrapolated to cover resistance to other types of small ruminant TSEs such as BSE or Atypical scrapie.

Based on the current limited knowledge provided by the study, it can only be concluded that breeding to increase the frequency of the addressed alleles may only confer relative resistance to the development of clinical disease caused by the current prevailing agents of TSE in Cyprus. Moreover, in terms of human and animal exposure to TSE agents, it remains unclear whether this selection will be efficient or not. This is because currently, there is no indication on the distribution of PrP^{Sc} or infectivity in N146, D146 or S146, H154 or R154 *PRNP* homozygous/heterozygous animals.

The BIOHAZ Panel concluded that the study conducted in Cyprus brings additional proof of a potential lower susceptibility to Classical scrapie in goats in H154, D146 and S146 *PRNP* allele carriers. It can be considered as encouraging information on the path for identifying *PRNP* polymorphisms that could be used as part of a genetic strategy to control and eradicate TSE agents in goats. However, the study on its own is an insufficient basis to evaluate accurately and reliably the efficacy and the potential adverse consequence of the large-scale breeding for H154, D146 and S146 *PRNP* alleles as a tool to control and eradicate Classical scrapie in Cyprus.

The BIOHAZ Panel recommends new investigations in order to assess the efficacy of breeding for the *PRNP* alleles addressed in the study as a mean to control and eradicate Classical scrapie in Cyprus goat populations. In addition, the operational possibility to conduct these alleles selection in the Cypriot goat population and the potential adverse effect of such selection on genetic variability in the Cypriot goat breeds should be evaluated.

Key words: Goat, TSE, Classical scrapie, genetic resistance, breeding program, Cyprus.

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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

It is scientifically recognised since several years that some polymorphisms of the *PRNP* gene are associated with differences in the phenotypic expression of prion diseases in sheep (incubation period, physiopathology and clinical signs). This association has led to the development at EU level of breeding programs based on the selection of animals known to be genetically resistant to TSE and to the implementation of eradication measures in TSE infected flocks based on a selective elimination of genetically susceptible animals. The appropriateness of these measures has been confirmed in the EFSA opinion on the breeding program for TSE resistance in sheep[2].

In goats, the association of genetic variability of *PRNP* with resistance or susceptibility to TSE, and in particular to Classical scrapie, remains unknown. However, the Cypriot authorities have recently sent to DG SANCO the final results of a Commission funded pilot project study[3] conducted in Cyprus and which has been just submitted for publication. These results indicate that polymorphisms of the *PRNP* gene at codons 146 and 154 could be associated with resistance/susceptibility to Classical scrapie in goats in Cyprus. If confirmed, these results could be very interesting in view of a possible future EU policy as regards scrapie control measures in goats.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

EFSA is requested to provide an opinion on the scientific validity of the Cypriot study and to indicate to what extent the information contained in this study can be used as relevant tools to control Classical scrapie and other TSE agents in goats in:

- Cyprus;
- Other EU Member States.

Clarification of the Terms of Reference:

Following consultation with the EU Commission Services, the terms of reference were amended and left as follows:

- To assess the scientific validity of the study;
- To indicate to what extent and based in this study genetic breeding can be used as a control program for Classical scrapie in goats in Cyprus.

A second EFSA opinion, to be delivered by the end of 2009, will address genetic resistance as a relevant tool for breeding for resistance to all TSEs of goats (including Atypical scrapie and BSE) in all the EU Member States (except for Classical scrapie in Cyprus).

ACKNOWLEDGEMENTS

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[2] The EFSA Journal (2006) 382, 1-46
[3] Included as a separate document.

The BIOHAZ panel members would like to acknowledge Dr. Penelope Papasavva-Stylianou and Dr. Otto Windl for their availability when clarifying certain aspects related to the findings of the Cypriot study and for kindly providing the raw data of the study for further analysis.

ASSESSMENT

1. Introduction

The objective of this assessment is to evaluate both the scientific validity of a Cypriot study on genetic TSE resistance in goats and its applicability in a breeding program aiming at eradicating Classical scrapie in goats in Cyprus. The study has been funded by the European Commission and was conducted by the Cypriot authorities under the supervision of the Community Reference Laboratory for TSEs (VLA-Weybridge (UK)), which has contributed in the design of the whole strategy, the evaluation of the results and the writing of the final report.

In the assessment included in here, a review of the available scientific literature on genetic TSE resistance in goats has been carried out. Following this, the Cypriot study has been reviewed and evaluated with detail. As part of this evaluation, the raw data of the study (kindly made available by the author) has been re-analysed in order to statistically assess the effect of different factors (*i.e.* breed, flock and genotype). This is presented in Appendix C.

The applicability of a control programme for Classical scrapie in goats in Cyprus, based on the Cypriot study, is discussed before the final conclusions and recommendations.

2. Overview of current scientific knowledge on genetic TSE resistance in goats.

2.1. TSE in goats.

The first observations of natural scrapie in sheep date back to 1732 in England and to 1759 in Germany (Laplanche *et al.,* 1999). In the following centuries, scrapie endemically affected flocks in several countries. It was reported for the first time in a goat living in a sheep flock where the disease had prevailed for several years (Chelle *et al.,* 1942). Since then, clinical scrapie cases in goats have been recorded throughout Europe and in other regions of the world. Although scrapie in goats is often found in mixed herds with sheep it has been observed to spread also from goat to goat (Hourrigan *et al*., 1979).

The first experimental challenges of goats with sheep scrapie showed 100% susceptibility and suggested that goats were highly susceptible to scrapie (Pattison *et al*., 1959, and Cuillé and Chelle, 1969). This contrasted to the results of similar experiments in sheep where survivors were regularly observed (Gordon *et al.*, 1966).

One confirmed case of natural BSE in a goat was discovered in France in 2002 (Eloit, *et al.,* 2005) and a number of BSE suspected cases in a goats in UK. Some of these were not confirmed during a retrospective study (Dustan *et al.*, 2008), but for one suspected case (Jeffrey *et al*., 2006) the results are still pending further outcome of the ongoing bioassays. Atypical scrapie in goats was recently recognised (Benestad *et al.,* 2003) as large number of asymptomatic goats began to be tested using more sensitive PrP^{Sc} detection methods with at least two distinguished molecular profiles, Nor98 and CH1641-like respectively (Baron *et al.*, 2007).

In the first five years of active surveillance more than 1 million goats were tested with 3,297 positive cases in EU countries (see Appendix A). More than 85 % of these cases have occurred in Cyprus. Until 2007, 661 cases were found in 'healthy animals' slaughtered for human consumption, of these, 33 cases were considered Atypical (EC, 2008).

2.2. Goat *PRNP* gene variability

Goats (and sheep) show a high degree of variation of the PrP^C protein gene (*PRNP*) coding sequence, with several synonymous as well as non-synonymous polymorphisms. In the past decade more investigation of *PRNP* variability in goats from Europe, Asia and America led to the discovery of variations reported below in Table 1.

Table 1. **Reported variations of the goat *PRNP* gene (silent mutations in italics).**

***PRNP* variation**[3]	**Detected in EU countries**	**Detected in non-EU countries**	**First Reference**
R143	GBR, ITA, GRE, *NL	USA, CHN, JPN	Goldmann *et al*., 1996
S138	GBR, ITA, FRA, CYP, GRE	USA, CHN, JPN, PAK	Goldmann *et al*., 1996
M142	GBR,FRA, SPN	USA, JPN	Goldmann *et al*., 1996
P42	GBR, ITA, FRA, GRE	USA, CHN, JPN, PAK	Goldmann *et al*., 1996
P240	ITA, FRA, GBR, GRE, SPN, CYP, *NL	USA,CHN, JPN, PAK	Goldmann *et al*., 1996
G102	GBR	CHN,JPN	Goldmann *et al*., 1998
G102 + 3 instead of 5 octarepeats	GBR		Goldmann *et al*., 1998
Q211	GBR, FRA, SPN	USA, CHN, JPN	Wopfner *et al*., 1999
A21	GRE,		Billinis *et al*., 2002
P23	GRE		Billinis *et al*., 2002
H220	FRA, GBR, CYP		Billinis *et al*., 2002
S49	GRE		Billinis *et al*., 2002
K107	GRE		Billinis *et al*., 2002
K 207	GRE		Billinis *et al*., 2002
H154[4]	GRE, ITA, SPN, CYP, FRA	USA, CHN	Billinis *et al*., 2002
Q168	ITA, GRE, CYP		Billinis *et al*., 2002
K222	ITA, FRA, GBR, SPN	USA, CHN	Agrimi *et al*., 2003
V37	ITA		Agrimi *et al*., 2003
P110	ITA		Agrimi *et al*., 2003
S127	ITA, GBR, SPN, FRA	USA, CHN, JPN	Zhang *et al*., 2004
L218		CHN	Zhang *et al*., 2004
R231		CHN	Zhang *et al*., 2004
S146	CYP	USA, CHN, JPN	Zhang *et al*., 2004; Kurosaki *et al*., 2005
Q133	ITA		Acutis *et al*., 2006
I137	ITA		Acutis *et al*., 2006
T202	ITA		Acutis *et al*., 2006
T219	ITA		Vaccari *et al*., 2006
G232	ITA		Vaccari *et al*., 2006
R211	FRA		Barillet *et al*., 2006
V179	CYP		Papasavva-Stylianou *et al*., 2007
D181	CYP		Papasavva-Stylianou *et al*., 2007
D146	CYP		Papasavva-Stylianou *et al*., 2007
H151	CYP		Papasavva-Stylianou *et al*., 2007
P194	ITA		Acutis *et al*., 2008
T142	ITA		Acutis *et al*., 2008
G211		CHN	Zhou *et al*., 2008
V125		CHN	Zhou *et al*., 2008
I219	SPN	CHN	Zhou *et al*., 2008

CHN=China; CYP= Cyprus; FRA= France; GBR=Great Britain; GRE=Greece; JPN=Japan; ITA=Italy; NL=The Netherlands; PAK=Pakistan; SPN=Spain; USA=United States of America;

[4] This denotes the amino-acid and its location in the polypeptide chain. For example, H = Histidine in the IUPAC single letter code for amino-acids; 154 indicates codon 154 of the caprine prion protein gene.

The most common polymorphism is S240P, which leads to the presence in goat populations of two highly frequent haplotypes: one with P240 and one with S240, the latter being identical in aminoacid sequences to the ovine *wild-type* (ARQ) PrP^{C}. The other alleles seem to have arisen by mutations on the background of these two central haplotypes, being generally related to one of them by a single amino acid substitution. Some exceptions are given by mutations M142 and H154, which have been found in linkage with both S240 and P240 (Goldmann *et al*., 1998; White *et al*., 2008; Billinis *et al*., 2002; Acutis *et al*., 2006). Furthermore two haplotypes with a double mutation (V37K222 and H154K222) have been reported (Vaccari *et al*., 2006; Colussi *et al*., 2008). These last alleles might derive from recombination events.

2.3. Association with susceptibility to disease or to infection

Scrapie association studies in goats are far more limited than those in sheep. Data available in goats are mainly resulting from cases control studies carried out in field infected flocks. It usually involved only a limited number of animals (in particular positive animals), and flocks (limited range of TSE agents).

In most (but not all) of these case control studies, the investigated event of interest was the number of clinical scrapie cases or the presence of abnormal PrP^{Sc} in the obex of the studied animals at culling. Because particularities in the TSE pathogenesis (long incubation period, late neuro-invasion), such information is not sufficient for assessing the infectious status of the investigated individuals. Consequently, rather than truly measuring the susceptibility / resistance to TSE infection that are associated to particular *PRNP* allele or genotype, those studies only provide a measurement of their protective effect on the clinical incidence of the disease within the context of the flock (limited life time of individual in the flocks – variable infectious pressure).

The measurement of this protective effect is considered to be an efficient tool for the identification of *PRNP* alleles and genotypes that could provide a higher resistance towards TSE agents' infection. However, they remain insufficient on their own to establish unambiguously such resistance to TSE infection.

Within these methodological limits, some caprine *PRNP* alleles were reported to be associated with increased resistance to the disease. A detailed review of both experimental and case control studies reporting on these is included in a table in Appendix B.

2.4. Polymorphic variants and its presence in different goat breeds

As investigation into the relationships between caprine *PRNP* haplotypes and absence of disease continues, knowledge of the *PRNP* genotype distribution in goat breeds has become increasingly important. Estimating the frequency of candidate alleles in a population is a preliminary step in understanding the feasibility of a selection programme to reduce the incidence of disease. Studies on *PRNP* allele frequencies in goats in Italy, France, USA, Japan, China (Zhang *et al*., 2004; Kurosaki *et al*., 2005; Acutis *et al*., 2008; White *et al*., 2008; Barillet *et al*., 2009) have been published so far. Table 2 shows the minimum and maximum frequencies reported for the polymorphic variants associated with a relative absence of scrapie in a population.

Table 2. **Summary of minimum and maximum frequencies for the polymorphic variants associated with some resistance to scrapie reported in literature**[5]

	Country (n of breeds)				
Polymorphism	**Italy (8)**	**France (2)**	**USA (10)**	**Japan (1)**	**China (5)**
I142M	0.0 28.2 (5/8)*	3.9 8.7 (2/2)	0.0 43.2 (4/10)	4.6	0.0
H143R	0.0 5.4 (4/8)	0.0	0.0 10.9 (4/10)	3.0	14.3 57.1 (5/5)
N146S/D	0.0	0.0	0.0 35.2 (7/10)	1.7	0.0 57.4 (1/5)
R154H	0.0 11.3 (6/8)	0.5 5.4 (2/2)	0.0 1.8 (1/10)	0.0	7.4 46.6 (5/5)
Q211R	0.0 13.7 (4/8)	7.1 18.5 (2/2)	0.0 9.7 (5/10)	12.7	0.0
Q222K	1.3 17.2 (8/8)	4.9 7.5 (2/2)	0.0 5.4 (2/10)	0.0	0.0 21.7 (1/5)

*number of breeds in which the polymorphism has been found out of the total breeds included in the study

What is clear is that there is an uneven distribution of alleles in the world goat population with the absence of some polymorphisms in some countries (*e.g.* N146S and N146D in Italy) or in some breeds (*e.g*. I142M found only in dairy breeds). The difference in allele distribution according to both breeds and geography has been highlighted in the Italian study in which *PRNP* gene variability has been investigated in breeds from Northern and Southern Italy. Significant differences in frequencies of some alleles were found between the two groups: for instances, H154 and K222 were more frequent in the Southern Italian goats. A genetic distance analysis showed that breeds belonging to the same geographical location clustered tightly. Caňòn *et al*. (2006) have previously pointed out the fact that the phylo-geographical structure of goat populations is more obvious than in other domestic species. In fact, gene flow among breeds has been restricted by spatial isolation, whereas the use of herd books is rarely practiced, so that geographical clines are maintained that predate breeds formation.

2.5. Conclusions

The limited studies carried out so far indicate that *PRNP* polymorphisms can modulate scrapie susceptibility in goats. Some polymorphisms could be associated with a lower susceptibility to TSE and are possible candidates for future breeding programmes. It has to be clarified whether the association of *PRNP* polymorphisms with apparent resistance to scrapie in goat is limited to those scrapie strains involved in the outbreaks studied and if this apparent resistance can be extended to other combinations of prion strains/goat breeds. Thus, more case-control studies in different countries and breeds are needed to "validate" these associations, along with experimental transmission studies investigating the degree of resistance (*e.g.* delay of the

[5] Taken from: Zhang *et al*., 2004; Kurosaki *et al*., 2005; Acutis *et al*., 2008; White *et al*., 2008; Barillet *et al*., 2009.

incubation period; resistance to clinical disease; resistance to infection) and the interaction with different TSE agents (*e.g.* BSE and Atypical scrapie).

The distribution of *PRNP* polymorphisms in goat populations varies widely in different breeds and countries and this heterogeneity will affect the ease and practicality of breeding programmes aimed at reducing the level of disease within a particular country or area. .

3. Review of the report on the Cypriot pilot project

3.1. Description of the Cypriot pilot project

The Cypriot study consists out of three parts and covers two years. In year one, a case control study (part one) was carried out. In year two an additional study on only scrapie positive animals was carried out (part two) in parallel with a breed survey (part three).

Year one of the study (1.1.2006-31.12.2006)

In part one, the case control study was carried out on 717 goats, including 218 scrapie-positive, 280 scrapie-negative and 219 healthy controls from 75 different scrapie-affected herds. The majority of the animals came from the Damascus breed and/or crossbred Damascus with local breeds. All the 717 samples of the case-control study were analyzed by DNA sequencing (Agrobiogen Laboratory, Germany).

Year two of the study (1.1.2007-31.12.2007)

In part two, a further 439 scrapie-positive goats derived from 120 different scrapie-affected herds were studied. Thirty six of these herds were already part of the case-control study. These 439 scrapie-positive animals are animals tested in addition to the 218 scrapie-positive goats of pat one of the study, *i.e.* the case-control study.

In part three (breed survey) blood samples were collected from 504 goats, derived from six different herds and belonging to the five main goat breeds in Cyprus (Damascus n=100, Local Machaeras n=104, Saanen n=100, Local Akamas n=100 and French Alpine n=100). The herd with Damascus goats is scrapie-affected. The goats of Machaeras local breed were derived from two herds, one of which is governmental and scrapie-affected. In addition, a total of 9615 samples from 25 different herds around Cyprus where scrapie has never been reported were also tested.

All the blood samples of the second year of the project, (439 scrapie - positive goats, 504 goats from the additional breed survey and 9615 goats from scrapie-free herds), were examined by single nucleotide polymorphism (SNP) analysis (Medigenomix Laboratory, Germany) in order to determine only the polymorphisms of codon 146 of the *PRNP* gene.

Results as presented in the report

Year one of the study:

The report as prepared by the researchers presents for year one, a case control study (part one) in Cypriot goat herds aiming at the identification of the effect of *PRNP* polymorphisms on TSE susceptibility. According to the author, the results obtained would indicate a higher

resistance to clinical disease in goats bearing the H154[6], D146 or S146 polymorphism than in goats bearing R154 or N146.

This case control study expands on results of a previous publication (Papasavva-Stylianou *et al.*, 2007) reporting preliminary data on two novel PrP^C protein gene polymorphisms at codon 146 in Cypriot goats which were only found in animals not affected by scrapie. The present case control study determines the sequence of the *PRNP* ORF of 717 goats, including 218 scrapie-positive, 280 scrapie-negative and 219 healthy controls from 75 different scrapie-affected herds. The majority of the animals came from the Damascus breed and/or crossbred Damascus with local breeds.

The definition of the three categories used in the case control study was as follows:

(a) The scrapie-positive goats were animals with clinical symptoms of scrapie and were confirmed positive after histological and biochemical examination of the brain (rapid or discriminatory testing).

(b) The scrapie-negative goats were animals with clinical symptoms similar to scrapie but confirmed negative after histological examination and/or rapid testing of the brain.

(c) The healthy control goats had no clinical symptoms and, as with the scrapie-negative animals, they were herd matched and, whenever this was possible, age, breed and sex matched with scrapie-positive animals.

With respect to codon 154, only 4 out of 218 (1.8%) scrapie-positive animals had the H154 allele. Twenty six out of 280 scrapie-negative controls, and 25 out of 219 healthy controls had the H154 allele, showing a frequency about ten times higher of H154 among the scrapie-negative and healthy controls than that in the scrapie-affected goats.

The author concludes that the two allelic variants (D146 or S146) were about ten times more frequently found among unaffected than scrapie-positive animals. However, 5 out of 218 scrapie-positive animals were heterozygous for one of the two "candidate" alleles at codon 146 (D146 or S146).

Year two of the study:

In the second year of the study, part one consists of testing of 439 further scrapie-positive animals revealed only one goat with one of the two "candidate" alleles. These 439 scrapie-positive goats derived from 120 different scrapie-affected herds. All these 439 goats had clinical symptoms of scrapie and were confirmed positive after histological examination and rapid testing of the brain. Of the 439 goats, 437 (99.5%) were homozygous (NN) at codon 146 and only one single goat (0.2%) was heterozygous (NS) at the same codon. The remaining one goat (0.2%) was found to have three alleles (N146, D146 and S146). None of the scrapie-positive goats was found to carry the allele D146. Similarly to the data obtained during case-control study, the data of SNP analysis of codon 146 of the scrapie-positive cases revealed that the allele N146 had an association with natural scrapie. In contrast, the alleles D146 and S146 showed high association with an absence of the disease.

In part two of the second year, in an additional breed survey of the distribution of these alleles in Cypriot herds, the two allelic variants were found in 19,5% and 8,5%, respectively, of 100 Damascus breed goats, but in none of 100 each of Saanen, Akamas and Machaeras Local, and French Alpine breed goats. In part two of the second year, also 9615 goats from "scrapie-

[6] This denotes the amino-acid and its location in the polypeptide chain. H = Histidine in the IUPAC single letter code for amino-acids; 154 indicates codon 154 of the caprine prion protein gene.

free" Cypriot herds were studied and had a SNP analysis for codon 146. The frequencies of the D146 or S146 alleles were found in 6.9% and 7.1%, respectively.

3.2. Critical review of the Cypriot pilot project

3.2.1. Analysis of the raw data of the Cypriot pilot project

As the age at observations was not considered in the statistical analysis of the pilot project, and may differ between case and control, the raw data of the study, which was kindly provided by Dr Papasavva-Stylianou, was re-analysed with survival analysis technique (see Appendix C for the detailed description of the re-analysis). This technique hypothesises that all the animals facing scrapie infection should succumb from this disease, with differences in incubation time between genotypes, the observation (age at death) concerning the control animals being described as "censored" and are modelled consequently.

The effect of the *PRNP* genotype was:

- Not significant for the codon 240.
- Poorly significant for the codon 102.
- Highly significant for the codons 154 and 146. The Odds Ratio (OR) for codon 146 gave a 6 to 10 lower risk for the ND or NS than for the NN genotype.

It must be explained that in this context, the meaning of the terms significant /not significant/ poorly or highly significant refers to the interpretation of p values as follows: $p>0.1$, not significant; $p\leq0.1$, poorly significant; $p\leq0.05$, significant; $p\leq0.01$, highly significant. However, some variation can be observed between the compared groups for a given codon, as detailed in Table 1 in Appendix C..

This re-analysis was in broad agreement with the initial analysis presented in the report. However, this can not be extended to infer an association between H154, D146 and S146 and resistance to infection, nor could it be extrapolated to cover resistance to other types of small ruminant TSEs such as BSE or Atypical scrapie. The rationale for these concerns is given below.

3.2.2. Absence of disease is not absence of infection

TSE incubation periods in sheep and goats can be long and apparently healthy animals can incubate the disease (Reckzeh *et al.*, 2007) and disseminate the infectious agent to other individuals or environment (Lacroux *et al.*, 2007 and 2008).

Due to the wide and early distribution of the TSE agent (long before the neuro-invasion phase of the disease) in peripheral organs of infected small ruminants, apparently healthy but incubating animals represent a risk of exposure for consumers. As previously addressed in an EFSA scientific opinion (EFSA, 2008), the lack of clinical disease or the absence of PrP^{Sc} in the central nervous system of a small ruminant does not warrant that an individual is not a source of TSE agent.

In the Cypriot study, investigation of the obex for vacuolar change or PrP^{Sc} deposition in the two control groups (scrapie-negative, healthy animals) is not sufficient to qualify the negative status of an individual., as further information would be needed for this(e.g. analysis of peripheral lymphoid tissue). If H154, D146 and/or S146 carriers can replicate TSE agents in

their peripheral tissues then they become a potential source of exposure to other animals and humans.

Indeed, based on our current limited knowledge, we can only conclude that breeding to increase the frequency of the highlighted alleles (H154, D146 and S146) may only confer relative resistance to the development of clinical disease caused by the current prevailing agents of TSE in Cyprus. Moreover, in terms of human and animal exposure to TSE agents (*e.g.* through body fluids, peripheral tissues) it remains unclear whether this selection will be efficient or not. There is no indication on the distribution of PrP^{Sc} or infectivity in 146N, 146D or 146S, 154H or 154R homozygous/heterozygous animal.

3.2.3. Limited information of the types of TSE in Cypriot goats

Of the several hundred Cypriot goat TSE cases, very few have been further investigated by PrP^{Sc} immunohistochemistry or western blotting and so little information is available on the potential diversity of prion diseases in these animals. According to the Cypriot authorities, 26 index cases have been referred to the EU Community Reference Laboratory for discriminatory western blotting according with Reg. (EC) 999/2001 (as amended) , including 2 H154 carriers.

3.2.4. Genetic structure of the goat Cypriot population

Evaluating the frequency of the "candidate" polymorphisms in the main breed of Cyprus goat population is a key element in any strategy aimed at their selection by breeding. However, the Cypriot study has only looked at a limited number of flocks. Working in a few flocks can be misleading since the particular choice of male goats by a breeder can lead to a "founder effect".

A better approach would be based on the selection of animals representing the variability of each breed in the whole population and the selection of a number of animals from each breed representing the demography of the global population. This selection should be based on the evaluation of Cyprian breeding system (genetic selection – use of artificial insemination (AI) versus natural mating).

The best results are generally obtained by selecting the samples in indexed bucks from each breed used over the last past years in the flocks. Without this country/region specific detail, it is very difficult to estimate the efficiency or effectiveness of any selection strategy.

4. Applicability of a control programme for Classical scrapie in goats in Cyprus based on the Cypriot case control study

The value of the presented scientific study is that it provides new data on the distribution of *PRNP* genotypes in goats in Cyprus with an important number of investigations, and thus expands our limited knowledge on *PRNP* variability in goats.

However, mainly due to its character as a field study, there are a number of significant gaps with regard to definition of the categories of animals investigated (see section 3.1), the TSE strains involved, and the status of the individuals. Given these limitations, it remains unclear to what level the reported allelic variants are protective or might delay incubation time.

The demonstration of the ARR allele effect in sheep on individuals TSE susceptibility and of the interest of breeding for ARR in the aim of controlling and eradicating Classical TSE

agents, required more than 10 years. It included not only multi-centric case control studies but also investigations on the effect of the ARR allele on the pathogenesis and transmission of TSE agents (including Classical scrapie agents and the BSE agent).

Taking advantage of the experience accumulated while investigating ARR allele effect in sheep, main aspects to be clarified with regards of the interest of H154, D146 and S146 *PRNP* polymorphism in goat as a tool for controlling Classical scrapie in the Cypriot goat population are listed in the sections below.

4.1. Effect of H154, D146 and S146 *PRNP* alleles on individual susceptibility to Classical scrapie infection

The data provided in the pilot project report are not sufficient to definitively determine the resistance / susceptibility level towards Classical scrapie associated to H154, D146 and S146 *PRNP* polymorphisms

Additionally, in sheep ARR allele provides a very strong resistance towards field Classical Scrapie: Virtually no infection seems to occur in naturally exposed ARR/ARR animals. However, in heterozygote ARR/ARQ clinical cases can be observed (odds ratio of 0.08 using ARQ/ARQ as a baseline) (Corbière *et al*., 2007). Because the low frequency of the alleles of interest in the Cypriot goat population it will remain extremely difficult through a field case control study to evaluate the susceptibility/resistance level of animals homozygous for different alleles.

In order to demonstrate unequivocally and measure the level of resistance towards Classical scrapie infection in goat harbouring *PRNP* 154 and 146 polymorphisms additional experiments could be carried out, including:

- Oral (around birth) and intracerebral challenge of goats homozygous and heterozygotes for the alleles of interest;
- Production, by appropriate mating, of animals harbouring the genotypes of interest in a number of affected herds and follow up evolution of these goats.

4.2. TSE agents diversity and resistance/susceptibility associated to H154, D146 and S146 *PRNP* alleles

TSE agents responsible for Classical Scrapie in small ruminants represent a mosaic of infectious agent harbouring distinct biological properties (see previous EFSA scientific opinion on certain aspects related to TSEs in small ruminants (EFSA, 2007)). Amongst the biological properties of TSE agents, the ability to develop and to transmit in individuals harbouring different *PRNP* background (polymorphisms) is Classically described (see previous EFSA scientific opinion on the breeding program for TSE resistance in sheep(EFSA, 2006)).

For instance, in sheep while ARQ variant is considered to be fairly susceptible to TSE in general, some particular Classical scrapie isolates (like SSBP-1) seem unable to propagate in ARQ/ARQ animals (Goldmann *et al*., 1994). Conversely, AHQ alleles seem to provide a certain level of resistance towards some TSE agents but is associated with fair susceptibility to experimental BSE (Foster *et al*., 2001) and increased risk to develop Atypical scrapie in both sheep (Moum *et al*., 2005) and goats (Colussi *et al*., 2008).

Asymptomatic cases with Atypical PrP^{Sc} distribution seem to be influenced by polymorphism on codon 154:RR at this codon appear to be linked with resistance to this type, 154:RH seems to be associated with susceptibility (Sofianidis *et al.,* 2008). However, in this study no breed is mentioned nor Western Blot patterns. These and possibly other types of scrapie, different from Classical scrapie in goats, can easily remain unidentified because of their particular features.

To date there is only very limited information on the potential diversity of TSE agents circulating in:

(i) affected flocks included in the presented pilot study;

(ii) small ruminants Cypriot population .

This paucity of information is a clear limitation for evaluating the interest of the identified alleles in the control Classical scrapie in Cypriot goat population.

In order to clarify this point several types of investigations would be helpful:

- Rational characterization (using biochemical and bioassay) of a panel of Cypriot TSE isolates;
- Experimental transmission of a panel of TSE isolates (including BSE and Atypical scrapie) in goats or other animal model (transgenic mouse model) harbouring the alleles of interest;
- In vitro conversion assays using a panel of TSE isolates.

4.3. Effect of H154, D146 and S146 *PRNP* alleles on Classical scrapie pathogenesis

Considering the data collected in the case control study, it remains unclear if the reported allelic variants are resistant to infection or might *e.g.* only delay incubation time, as reported for the M142 *PRNP* allele (Goldman *et al.*, 1996).

In order to scientifically assess this point, and in particular to (a) assess the potential effect of H154, D146 and S146 alleles on TSE agent distribution organism of affected individual (with clear consequences in term of exposure risk for consumer, and (b) to investigate the potential existence of an healthy carrier state in H154, D146 and S146 individuals, two different and complimentary approaches could be taken:

- A prospective study in Cyprus with systematic assessment of PrP^{Sc} presence in peripheral tissue of clinically healthy and scrapie affected animals from infected herds, in order to accumulate data on the potential distribution of TSE Agent in the organs of H154, D146 and S146 animals.
- A kinetic study of TSE agent dissemination in organs of orally challenged goats with Classical scrapie. Groups of wild-type and H154, D146 and S146 (homozygote and heterozygotes) individuals challenged around birth with Classical scrapie could be sequentially killed in order to identify peripheral and central tissue involvement during disease development (if any).

4.4. Effect of H154, D146 and S146 *PRNP* alleles on the dynamics of TSE agent transmission in affected flocks

The reproduction Ratio (R_0) (number of secondary cases generated by an infected individual) is a key factor determining the expansion or the fading of a disease in a population. The

reproduction ratio is the results of dynamic interactions between the host/ the infectious agent and the environment. In TSE context even in case of an imperfect individual resistance to infection (partial resistance to infection linked to particular *PRNP* polymorphism), the R_0 at flock level could be lower than 1, leading to protection in a homozygote herd.

The effect of the genetic distribution on the reproduction ratio of the infection (R_0) can not be quantified from the data provided and the effect of a breeding program on the transmission of the infection and the development of the epidemic cannot be evaluated.

To quantify the transmission of scrapie in goats, data that give information about exposure should be evaluated in combination with data about new infections in that same period. This usually requires data on infections/disease over a period of time preferably in combination with a good overview of the herds in which the data are collected. Typically, case-control data are not suitable for this question, since the background data on the full herd are lacking, thus the level of exposure which led to the case can not be estimated.

If the present data would be completed by giving a complete picture of the genetic distribution of the full herds for at least ten herds, a first step in this analysis can be made. For a higher confidence level, repeated screening over a few years will be sufficient. It would also be advisable to make a more complete TSE evaluation, by testing for TSEs on more tissues and/or using an ante-mortem test.

4.5. *PRNP* allele selection and adverse effect on production or health traits

Adverse effect on health and production traits associated with breeding for particular trait is a well recognised risk in genetics. Such potential risk was largely debated when considering the breeding for resistance policy in sheep (EFSA, 2006), but no adverse effect was observed. While a large number of studies and datasets were available for discussion of such risk in sheep, in goats data remain sparse.

The probability that an adverse effect would be associated to particular *PRNP* allele selection in goat species considered in general remains low. However, the risk of a "founder effect" should be considered in the particular situation of the Cypriot goat population, due to the low frequency of a potentially resistant allele (potential reduced genetic variability related to the population size).

4.6. Capacity for selection and diffusion of *PRNP* allele in Cypriot goat population

According to data available, the potential resistance alleles (H154, D146 and S146) in Cypriot goat population are represented at frequencies lower than 10% in all breed and absent in some of the investigated breeds. Because the caveats in the recruitment of the population samples the reliability of these figures is imperfect and new investigations should be carried out. They however suggest that 154 and 146 *PRNP* polymorphisms frequencies are low.

In such context, the organisation of goat breeding and genetic selection system in Cyprus will directly impact on the feasibility and duration of any *PRNP* selection scheme in producing goat population. To date, no data seem available with regards to these aspects.

If any *PRNP* selection programme should be considered in goat population in Cyprus or elsewhere, the capacity of breeding system to support the diffusion of particular *PRNP* alleles should be considered and modelled.

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

- The study conducted in Cyprus brings additional evidence of a potential lower susceptibility to Classical scrapie in goats in H154, D146 and S146 *PRNP* allele carriers. It can be considered as encouraging information on the path for identification of *PRNP* polymorphisms that could be used as part of a genetic strategy to control and eradicate TSE agents in goats.
- The present study on its own does not provide the basis for the accurate and reliable evaluation of the efficacy and of the potential adverse consequence of the large-scale breeding for H154, D146 and S146 *PRNP* alleles as a tool to control and eradicate Classical scrapie in Cyprus.

RECOMMENDATIONS

In order to assess the efficacy of breeding for H154, D146 and S146 *PRNP* alleles in goats as mean to control and eradicate Classical Scrapie in Cyprus goat populations new investigations are recommended:

- Characterization of the susceptibility/resistance towards TSE agents occurring in Cyprus.
- Impact of the H154, D146 and S146 *PRNP* alleles (heterozygous and homozygous animals) on TSE resistance and pathogenesis in naturally or experimentally infected goats;
- Capacity of H154, D146 and S146 *PRNP* allele carriers to transmit the infectious agent.
- In addition, the operational possibility to conduct the selection of H146, D146 and S146 *PRNP* alleles in the Cypriot goat population and to identify the potential adverse effects of such selection on genetic variability in the Cypriot goat breeds should be evaluated.

DOCUMENTATION PROVIDED TO EFSA

1. Pilot project report: “Polymorphisms of caprine PrP gene and their association with resistance or susceptibility to natural scrapie”. Dr. Penelope Papasavva-Stylianou. Published as a separate document.

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APPENDICES

APPENDIX A. YEARLY RESULTS OF TSE TESTING IN GOATS IN THE EU

	2002		2003		2004		2005		2006		2007		2008		TOTAL	
	N° tests	Positives	N° tests	Positives	N° tests	Positives	N° tests	Positives	N° tests	Positives	N° tests	Positives	N° tests	Positives	Total tests	Positives:
Austria	578	0	1,418	0	358	0	1,199	0	1,611	0	1,820	0	1,829	0	8,813	0
Belgium	151	0	120	0	272	0	908	0	1,063	0	754	0	167	0	3,465	0
Bulgaria	201	0	86	0	724	0	1,867	0	2,640	0	2,511	0	1,813	0	9,842	0
Cyprus	na	na	554	195	1,335	354	3,387	387	6,025	713	6,781	1,158	na	na	18,082	2,807
Czech Republic	na	na	274	0	86	0	216	0	113	0	163	0	271	0	1,123	0
Denmark	165	0	448	0	1,320	0	1,150	0	1,716	0	1,564	0	1,837	0	8,200	0
Estonia	na	na	0	0	0	0	17	0	61	0	55	0	11	0	144	0
Finland	245	4	250	0	261	0	830	4	516	0	431	0	273	0	2,806	8
France	28,370	18	27,359	19	6,923	27	149,986	32	162,822	15	179,644	7	65,839	20	620,943	138
Germany	1,656	0	4,839	0	5,742	0	4,667	0	4,604	0	3,969	0	3,655	0	29,132	0
Greece	9,505	9	7,100	19	3,628	15	4,585	35	7,081	22	5,880	53	2,255	22	40,034	175
Hungary	na	na	0	0	332	0	267	0	217	0	468	0	168	0	1,452	0
Ireland	1	0	1	0	1	0	79	0	208	0	156	0	135	0	581	0
Italy	3,701	9	5,226	6	3,654	2	28,528	9	27,916	17	24,514	6	13,941	1	107,480	50
Latvia	na	na	0	0	1	0	40	0	17	0	66	0	10	0	134	0
Lithuania	na	na	0	0	4	0	6	0	27	0	94	0	131	0	262	0
Luxembourg	0	0	56	0	77	0	210	0	450	0	533	0	310	0	1,636	0
Malta	na	na	0	0	34	0	65	0	47	0	9	0	48	0	203	0
Netherlands	4,052	0	5,110	0	620	0	20,160	0	25,583	0	15,770	0	578	0	71,873	0
Poland	na	na	0	0	0	0	0	0	167	0	717	0	934	0	1,818	0
Portugal	552	0	2,787	0	7,287	0	5,638	0	6,367	0	8,634	0	8,488	2	39,753	2
Romania	na	na	na	na	na	na	na	na	na	na	618	2	928	0	1,546	2
Slovakia	na	na	8	0	5	0	105	0	68	0	83	0	12	0	281	0
Slovenia	182	0	182	0	261	0	591	4	386	0	429	0	488	0	2,519	4
Spain	5,375	1	7,938	1	3,678	0	39,973	10	56,899	11	38,638	20	5,482	16	157,983	59
Sweden	78	0	125	0	89	0	266	0	248	0	86	0	55	0	947	0
United Kingdom	15	0	245	1	147	0	2,645	4	5,034	13	2,732	26	664	3	11,482	47
Total EU :	**54,827**	**41**	**64,126**	**241**	**36,839**	**398**	**267,385**	**485**	**311,886**	**791**	**297,119**	**1,272**	**110,322**	**64**	**1,155,992**	**3,297**

na = not available

Source: EC monthly TSE reports. Last updated: 29/01/2009. Available at: http://ec.europa.eu/food/food/biosafety/bse/mthly_gt_cml_reps_tse2002_en.pdf

APPENDIX B. EXPERIMENTAL AND CASE CONTROL STUDIES REPORTING ON *PRNP* ALLELES ASSOCIATED WITH RESISTANCE TO SCRAPIE

Type of study	PRNP variation	TSE agent	Exposure route	N of goats	Testing tissue targeted	Effect claimed by author(s)	Limitations	Reference
Experimental studies	142M	Sheep scrapie CH1641	Intra-cerebral (i.c.)	6	Brain tissue	Extended incubation period	- No infectivity study performed - No Lymphoreticular system tested	Goldmann *et al.*, 1996
			Subcutaneous	4				
		Sheep-passaged ME7 scrapie	i.c.	12				
		BSE agent	i.c.	9				
			Subcutaneous	5				
	102G	Sheep-passaged SSBP/1	i.c.	5	Brain tissue	Extended incubation period	- No infectivity study performed - No Lymphoreticular system tested	Goldmann *et al.*, 1998
Case control studies	143R 154H	Classical scrapie	n/a[7]	51	Cerebrum, brain stem, cerebellum	Resistant to clinical disease	- No infectivity study performed. - No Lymphoreticular system tested	Billinis *et al.*, 2002
	222K	Classical scrapie	n/a - Linked in some of the studied outbreaks to subcutaneous administration of a vaccine against contagious agalactia (*Mycoplasma agalactiae*))	177	Obex	Protective against scrapie	- No infectivity study performed - No Lymphoreticular system tested	Acutis *et al.*, 2006
	143R 154R/H (heterozygous)	Classical scrapie	n/a – Subcutaneous, linked to administration of a vaccine against contagious agalactia (*Mycoplasma agalactiae*))	100	Obex, Lymphoreticular system (tonsil, lymph nodes spleen, third eyelid)	Extended incubation period	- No infectivity study performed	Vaccari *et al.*, 2006

[7] n/a = Non applicable. These are scrapie cases naturally acquired. When further information is presented in the study on potential route, this is included in also included here.

Type of study	PRNP variation	TSE agent	Exposure route	N of goats	Testing tissue targeted	Effect claimed by author(s)	Limitations	Reference
Case control studies	222K	Classical scrapie	n/a (subcutaneous?) Linked to administration of a vaccine against contagious agalactia (*Mycoplasma agalactiae*))	100	Obex, Lymphoreticular system (tonsil, lymph nodes spleen, third eyelid)	Scrapie resistance	- No infectivity study performed	Vaccari *et al.*, 2006
	146S, D 154H	Classical scrapie	n/a	250	Brain tissue.	Protection against scrapie	- No infectivity study performed - No Lymphoreticular system tested	Papasavva-Stylianou *et al.*, 2007
	154H	Nor98	n/a	254	Obex.	Risk factor for Nor98 occurrence	- No infectivity study performed - No Lymphoreticular system tested	Colussi *et al.*, 2008
	222K	Classical Scrapie	n/a	254	Blood, spleen, ileum, mesenteric lymph nodes, tonsil and obex.	Protection against natural scrapie infection	- Positive when PrP^{Sc} present in at least one tissue, but not specified.	Barillet *et al.*, 2009
	154H	Classical scrapie	n/a	254	Blood, spleen, ileum, mesenteric lymph nodes, tonsil and obex.	Protection against natural scrapie infection	- Positive when PrP^{Sc} present in at least one tissue, but not specified.	Barillet *et al.*, 2009
	211Q	Classical scrapie	n/a	254	Blood, spleen, ileum, mesenteric lymph nodes, tonsil and obex.	Protection against natural scrapie infection	- Positive when PrP^{Sc} present in at least one tissue, but not specified.	Barillet *et al.*, 2009

APPENDIX C. STATISTICAL ANALYSIS OF THE RAW DATA FROM THE STUDY ON THE EFFECT *PRNP* POLYMORPHISMS ON SCRAPIE SUSCEPTIBILITY IN GOAT FROM CYPRUS

As the age at observations was not considered in the statistical analysis of the pilot project, and may differ between case and control, the raw data of the study, which was kindly provided by Dr. Papasavva-Stylianou, was re analysed with survival analysis technique. This technique hypothesizes that all the animals facing scrapie infection should succumb from this disease, with differences in incubation time between genotypes, the observation (age at death) concerning the control animals being described as "censored" and are modelled consequently.

1. Material and methods

Raw data as provided by the author of the report of the Cypriot study was employed for this analysis. This is presented in a separate Excel© file.

From the 12 *PRNP* polymorphisms found by the authors, 4 presented sufficient variability to be analysed (102, 146, 154 and 240).

The data were studied following the model:

Age at death = breed effect + herd effect + *PRNP* genotype + residual

In "survival analysis" it is hypothesized that all individuals may die from scrapie (whatever their genotype) and that non scrapie animals (dying for any other reasons, or killed for experimental purposes) were not given the chance to express their age at scrapie. These animals are considered in the analysis assuming their age at death is "censored".

The data were processed using the "survival kit" software (Ducrocq, V. and J. Sölkner, 1994. The Survival Kit - a Fortran package for the analysis of survival data. Proc. 5th World Congress on Genetics Applied to Livestock Production, 22 51-52.) The option "Cox" was used.

For each codon, three data sets were analysed:

- Scrapie positive vs. scrapie negative (pos. vs. neg.).
- Scrapie positive vs. healthy control (pos. vs. hel.).
- Scrapie positive vs. scrapie negative + healthy control (pos. vs. all).

2. Results

Results are presented in Table 1.

Table 1. **Results of the survival analysis including odds ratio for the different genotypes at 4 codons, with their confidence interval.**

			Prob. > X^2			Odds Ratio (OR) of allele combinations		
Codon	**Comparison***	**Nb. records**	**Breed**	**Flock**	***PRNP***	**WW**	**WG**	
102	pos. vs.neg.	492	0.15	0.98	0.10	1.000	0.480 (0.219 - 1.322)	
102	pos. vs. hel.	435	<0.01	0.97	0.04	1.000	0.299 (0.108 – 0.825)	
102	pos. vs. all	711	<0.01	0.96	0.01	1.000	0.332 (0.120 – 0.915)	
						NN	**ND**	**NS**
146	pos. vs.neg.	492	0.10	0.67	<0.01	1.000	0.184 (0.057 – 0.591)	0.125 (0.030 – 0.520)
146	pos. vs. hel.	435	0.01	0.54	<0.01	1.000	0.146 (0.046 – 0.466)	0.084 (0.020 – 0.349)
146	pos. vs. all	711	<0.01	0.41	<0.01	1.000	0.125 (0.039 – 0.401)	0.078 (0.019 – 0.322)
						RR	**RH**	
154	pos. vs.neg.	492	0.22	0.88	0.01	1.000	0.314 (0.114 – 0.864)	
154	pos. vs. hel.	435	<0.01	0.99	<0.01	1.000	0.235 (0.085 – 0.654)	
154	pos. vs. all	711	0.01	0.88	<0.01	1.000	0.218 (0.078 – 0.604)	
						SS	**PS**	**PP**
240	pos. vs.neg.	492	0.20	0.74	0.32	1.000	1.115 (0.665- 1.209)	0.768 (0.472 – 1.250)
240	pos. vs. hel.	435	<0.01	0.92	0.32	1.000	0.991 (0.735– 1.335)	1.445 (0.891 – 2.343)
240	pos. vs. all	711	0.01	0.95	0.83	1.000	1.063 (0.789 – 1.431)	1.136 (0.700 – 1.844)

(*) Scrapie positive vs. scrapie negative (pos. vs. neg.); Scrapie positive vs. healthy control (pos. vs. hel.); Scrapie positive vs. scrapie negative+ healthy control (pos. vs. all).

The results indicate that:

1. The Herd (mixed or caprine only) effect was never significant.

2. The Breed (Macheras, Damascus or crossbred) effect was significant most of the time.

3. The *PRNP* genotype was:

    - Not significant for the codon 240.
    - Poorly significant for the codon 102.
    - Highly significant for the codons 154 and146. The odd ratio for the codon 146 gave a 6 to 10 lower risk for the D or S carriers.

It must be explained that in this context, the meaning of the terms significant /not significant/ poorly or highly significant refers to the interpretation of p values as follows: $p>0.1$, not significant; $p\leq0.1$, poorly significant; $p\leq0.05$, significant; $p\leq0.01$, highly significant. However, some variation can be observed between the compared groups for a given codon, as detailed in Table 1.

On the whole, this analysis confirms the results presented by the authors.