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Pathological and epidemiological significance of *Goose haemorrhagic polyomavirus* (GHPV) infection in ducks

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Summary

Goose haemorrhagic polyomavirus (GHPV) is the viral agent of haemorrhagic nephritis enteritis of geese (HNEG), a lethal disease of goslings. It was recently shown that GHPV can also be detected in Muscovy and mule ducks. The goal of the present study was to investigate the pathobiology of GHPV in ducks.

In the first experiment, field isolates of GHPV from Muscovy or mule ducks were fully sequenced and compared to goose GHPV. These duck isolates were then used to inoculate one-day-old goslings. Typical clinical signs and lesions of HNEG were reproduced, indicating that “duck-GHPV” isolates are virulent in geese. In the second experiment, 1-day-old and 21-day-old Muscovy ducklings were infected by a reference GHPV strain. In both cases, neither clinical signs nor histopathological lesions were observed. However, the virus was detected in cloacal bursae and sera, and serological responses were detected at 12 days post infection.

These findings suggest firstly that one common genotype of GHPV circulates among ducks and geese and secondly that ducks may be infected by GHPV but show no pathologic evidence of infection, whereas geese express clinical signs. GHPV infection should therefore be considered as being carried in ducks and of epidemiological relevance in cases of contact with goose flocks.

Introduction

Goose haemorrhagic polyomavirus (GHPV) is the agent of haemorrhagic nephritis enteritis of geese (HNEG), a disease of young geese (*Anser anser*) causing high morbidity and mortality (Guerin *et al.*, 2000). Affected birds are commonly 4 to 12 weeks of age. Under field conditions, death is the most common outcome, generally preceded by coma (Guerin *et al.*, 2000). The *post mortem* findings are oedema of subcutaneous tissues, gelatinous ascites, inflammation of the kidneys and often haemorrhagic enteritis. Furthermore, GHPV infection induces immunosuppressive B-cell depletion in the cloacal bursa of these birds (Lacroux *et al.*, 2004).

It was recently shown that GHPV may be detected in Muscovy ducks (*Cairina moschata*) and mule ducks (hybrid from a Muscovy duck and a Pekin duck) displaying retarded growth or feathering disorder, and suffering from secondary infections and increased mortality (Pingret *et al.*, 2008). By analogy with infection in geese, Pingret *et al.* (2008) suggested that GHPV infection in ducks could be associated with a lymphoid depletion and contribute to immunodepression in ducklings. So far, no experimental data have characterized the GHPV isolates circulating among flocks of ducks.

In the present study, we investigated the genetic relationship of “duck-GHPVs” with the previously described goose isolates and their virulence when inoculated into goslings. We then evaluated the pathobiology of GHPV infection in ducks, based on pathological, virological and serological approaches. The pathological significance of GHPV infection in ducks was investigated in order to determine whether this avian polyomavirus should be considered as a novel putative immunosuppressive virus of ducks, along with parvoviruses and circoviruses.

Materials and Methods

Sampling of Duck-GHPV isolates. Three GHPV isolates from the spleen of naturally infected birds and stored at -80°C at the laboratory were submitted for molecular analyses at Scanelis Laboratory, France. The first sample was isolated from a naturally infected goose flock showing typical clinical signs of HNEG in 2008. The two other samples were isolated in 2008 from infected Muscovy and mule ducks examined because of increased mortality, growth retardation and feathering disorders, and submitted to the Analysis Department of Scanelis Laboratory for virological analysis (Pingret *et al.*, 2008). These samples were submitted to real-time quantitative PCR assays for GHPV, DuCV (*Duck circovirus*), DEV (*Duck enteritis virus*), DPV (*Duck parvovirus*) and GPV (*Goose parvovirus*), using standard procedures.

Genome sequencing and analysis. Four GHPV isolates, *i.e.* our reference strain (namely Toulouse Goose 2000), a 2008 goose isolate, a 2008 Muscovy duck isolate and a 2008 mule duck isolate were subjected to direct DNA extraction, using a silica-based extraction kit as recommended by the supplier (Macherey-Nagel, Hoerd, France). PCR amplification was performed using a set of 10 primers (Table 1), resulting in 5 overlapping PCR products. PCRs were performed following the Platinum Taq DNA polymerase supplier recommendations (Invitrogen Ltd, Paisley, UK) in a 50µl final volume. Cycling conditions were as follows: 95°C 2 min. / 40x [94°C 15 sec. / 55°C 20 sec. / 72°C 45 sec.] / 72°C 5 min.

Bioinformatics analyses (contigs assembly, sequences alignments) were performed using Vector NTI software® (Invitrogen Ltd, Paisley, UK) and primers were designed using the on-line software Primer-3 (Rozen & Skaletsky, 2000).

Inocula. Inocula were prepared by crushing spleens from the 3 natural cases described previously and then mixing the tissue in Dulbecco Minimum Eagle's Medium (DMEM). After centrifugation (15 min, 10000g), the supernatant was retrieved. A fourth inoculum was purified from a cell culture as previously described (Guerin *et al.*, 2000). GHPV titres were

determined by real-time quantitative PCR analysis as previously described (Gelfi *et al.*, 2010) and expressed as genome-equivalent viruses (*gev*).

DNA extraction and GHPV quantitative PCR. DNA extraction was performed using the *High Pure PCR template Preparation Kit* (Roche). The DNA suspension (200µl) was extracted from one sample (*i.e.* 35 mg of bursa or 200µl of serum). SYBR® Green Quantitative PCR amplification was performed using the set of primers F (5'-GATGGTGCTTATCCCGTGGA-3') and R (5'-TTCATTCCGGGATGGGTCT-3'), targeting the VP1 gene. All amplification reactions were performed in a total volume of 22µl. Each well contained 2µl extracted DNA sample or positive standard control, 12.5µl SYBR® Green Supermix (Biorad), 0.7µl of each primer (10 µM) and 6.1µl distilled water. The thermal profile consisted of a first step of denaturation (95°C for 15 min) and 40 cycles of two steps: 95°C for 15 sec and 60°C for 1 min. DNA quantification was determined using standard controls. Data were expressed as the log of the number of *gev* detected per µl of DNA extracted from 35 mg of bursa or from 1 ml of serum.

Experimental infections. GHPV infection of day-old goslings. Thirty-five day-old goslings were obtained from a local hatchery. Birds were housed according to the guidelines of the European Community on Animal Care (European Council directive 86/609/ECC, 24 November 1986) in wire-floored cages with infrared lamps for heating, and were provided with food and water *ad libitum*. At day 1, birds were divided into 5 groups (Table 2). Group 1 was the negative control group and group 2 was the positive control group (infection with the “Toulouse Goose 2000” strain). Goslings in the other groups were inoculated with viral suspensions prepared from naturally infected birds: “Toulouse Goose 2008” (group 3), “Toulouse Muscovy duck 2008” (group 4) and “Toulouse Mule duck 2008” (group 5). All birds were inoculated both subcutaneously (half of the infectious dose) and orally (half of the infectious dose), and then clinically monitored on a daily basis from day 1 to their death or

the end of the study (day 17). At the end of the study, all birds still alive were euthanized according to standard procedures.

GHPV infection of day-old ducklings. Fifty day-old Muscovy ducklings were obtained from a local hatchery, checked for GHPV antibodies using the ELISA described below, and raised in the conditions previously detailed. At day 1, birds were divided into 2 groups (25 birds per group). Group 1 was the negative control group (mock infected) and group 2 was the infected group (Toulouse Goose 2000 strain - 10^8 *gev* / bird). All birds were inoculated both subcutaneously (half of the infectious dose) and orally (half of the infectious dose), and then clinically monitored on a daily basis from day 1 to the end of the experiment. At 2, 5, 10, 20 and 30 days post-infection (dpi) 5 birds were randomly sampled in each group for euthanasia and necropsy. The experiment ended at 30 dpi.

GHPV infection of immunodepressed ducklings. Forty-five day-old Muscovy ducklings were obtained from a local hatchery and raised in the conditions previously described. They were checked for GHPV antibodies at 7 and 14 days of age, when they were divided into 5 groups (Table 3). Group 1 was the negative control group (mock infected). For groups 2 and 4, an immunodepression was chemically induced in birds at day 14 and day 19 by intramuscular injection of dimethylbutyrate of dexamethasone (Dexamedium®) at a dose of 1.4 mg / kg according to a procedure developed by Fowles *et al.* (1993). Then, birds in groups 3 and 4 were infected at day 21 by subcutaneous injection (half of the infectious dose) and the oral route (half of the infectious dose) with the Toulouse Goose 2000 strain (10^8 *gev* / bird). Thus, group 2 was the immunodepression control group (dexamethasone only) and group 3 was the infection control group (GHPV infection only). Birds in group 4 were immunodepressed and GHPV infected. Finally, group 5 comprised non-infected sentinel ducklings (5 birds) placed at day 21 in the same cage as the infected birds. All ducklings were monitored daily for clinical signs from day 14 to the end of the experiment. At 5 and 12 dpi, birds were randomly chosen from each group for euthanasia and necropsy.

Weight monitoring, gross pathology and sampling. For the GHPV infection of day-old goslings, after natural death or euthanasia, each bird was necropsied for observation of HNEG gross lesions. Kidney, bursa, duodenum and jejunum were sampled and processed for histopathology. For the GHPV infection of day-old ducklings, the same procedure was applied to euthanized ducklings and samples of bursa and serum were stored at -20°C for virological analysis.

For the GHPV infection of immunodepressed ducklings, individual weights of each duckling were monitored at day 21 (5 dpi) and before euthanasia (12 dpi). During lethal exsanguination, a blood sample was collected from each bird for virological and serological monitoring. After necropsy, two samples were collected from the cloacal bursa for histological examination and virological analysis. Samples of kidney, duodenum and jejunum were also collected for histological examination. Finally, for the non-immunodepressed ducklings killed at 12 dpi, the cloacal bursa was carefully removed and the bursa to body weight ratio calculated.

Serology. An indirect ELISA was developed by adapting procedures routinely used in the laboratory (Gelfi *et al.*, 1999). Briefly, antigen was prepared from semi-purified viral particles. A 96-well microtitre plate was sensitised overnight with antigen, blocked with gelatin for 1h, washed in PBS-tween 0.05%, incubated with test serum, washed 3 times in PBS-tween 0.05 %, then incubated with a secondary goat anti-duck antibody bound with alkaline phosphatase (KPL). After 3 washes in PBS-tween (0.05 %) and one wash in PBS, the plates were read and the optic density (OD) was determined for each well.

Quantitative ELISA titres were expressed as the inverse of the dilution giving an OD value three times as great as the OD value of the standard negative control, according to standard procedures (Gelfi *et al.*, 2010). A titre of less than 20 was considered negative. For semi quantitative results, samples are tested at 1:20 and ELISA titres obtained according with the formula: $OD \text{ sample} - OD \text{ Negative Control} / OD \text{ Positive Control}$.

Histopathology. Tissues samples collected during necropsy were fixed in 10% buffered formaldehyde solution, then routinely processed, embedded in paraffin wax, sectioned and stained with haematoxylin and eosin.

Statistical analyses. All the statistical analyses of weights, viral loads and serological titres were performed after a log transformation of the data. The Student test was used for each analysis and $p < 0.05$ was considered statistically significant.

Results

Genomic analysis of field duck-GHPV isolates. To avoid any genetic change induced by propagation in cell culture, a direct PCR cloning strategy was applied on splenic tissue of geese and ducks. A set of overlapping primers (Table 1) was used to amplify the entire genome. After sequencing of the PCR products, the genomes were assembled and analysed using Vector NTI. A multiple alignment of the whole genomes was performed in order to identify single nucleotide polymorphisms (SNP). The genome sizes range from 5252 to 5254 bp. By comparison with the whole genome sequence of the Germany 2001 isolate (GenBank Accession number AY140894; Johne & Muller, 2003), 8 to 15 nucleotide changes could be identified on the complete sequence of our 4 GHPV genomes. Very few amino acid changes could be identified, several of which were between similar amino acids (i.e. Serine to Threonine, or Glycine to Alanine) (Table 4). None of them could be associated to either a duck or a goose host. Furthermore, no change could be identified on the VP1, or on the large T or small t antigens of any of the genomes. Altogether, these data strongly suggest that a common genotype of GHPV infects both geese and ducks.

The complete genome sequences of viruses “Toulouse Goose 2008”, “Toulouse Goose 2000”, “Toulouse Muscovy Duck 2008” and “Toulouse Mule Duck 2008” were

deposited in GenBank under accession numbers HQ681902, HQ681903, HQ681903 and HQ681903, respectively.

Reproduction of HNEG in goslings infected with duck-GHPV isolates. When infected by duck GHPV isolates, all goslings except two died within 5 to 16 days with haemorrhagic diarrhoea followed by prostration then coma prior to death. Only one gosling infected with a Muscovy duck GHPV (group 4), and one gosling infected with a mule duck GHPV (group 5) survived until day 17. Birds from the control group did not present clinical signs, and were euthanized at day 17. At necropsy, all diseased goslings presented characteristic gross lesions of HNEG (Lacroux *et al.*, 2004): haemorrhagic enteritis, ascites, nephritis or haemorrhagic nephritis. The bird surviving from the infection by the Muscovy duck isolate (group 4) presented haemorrhagic enteritis lesions (Table 2). Microscopic lesions observed in affected goslings were characteristic of HNEG (Lacroux *et al.*, 2004): necrotic-haemorrhagic foci on the duodenum and jejunum, degenerative epithelial tubular lesions on the kidney and marked pycnosis on the medulla area of bursal lobules. Birds from the control group did not present any microscopic lesions.

GHPV infection of day-old Muscovy ducklings. Serological analyses of commercial day-old Muscovy ducklings demonstrated that these birds were GHPV antibody-free. Thus an experimental infection of these birds was performed at one day of age with the Toulouse Goose 2000 GHPV strain. No clinical signs were observed in the infected ducklings on any day. At necropsy, no gross lesions of any organ were observed. Histological analysis of bursa, kidney, duodenum and jejunum did not reveal lesions. However, from each sampling day from day 2 to day 30 post infection, GHPV was detected by PCR in the bursa and the serum of all infected ducklings (data not shown), suggesting an active replication of the virus.

GHPV infection of immunodepressed Muscovy ducklings. During the experiment, GHPV antibodies were detected in ducklings at 7 days of age, before the experimental infection,

(data not shown) while no virus could be found. At 14 days, these antibodies had disappeared, suggesting that they were maternally derived. No clinical signs were seen before day 14 (dexamethasone injection) in any of the birds. After dexamethasone injection, significant growth retardation was observed in the treated birds (data not shown). The birds did not present any other clinical signs before infection. After viral inoculation, no clinical signs were observed during the entire experiment. Statistical tests did not show significant effects of GHPV infection on body weight of either non-immunodepressed birds at 5 dpi ($p = 0.62$) or 12 dpi ($p = 0.42$), or immunodepressed birds ($p = 0.85$ and $p = 0.78$, respectively). At necropsy, gross lesions of aspergillosis were observed in the airsacs and lungs of immunodepressed birds, without relation to their GHPV infectious status. Moreover, the lymphoid bursae of all of these birds appeared atrophied and fibrous (four times smaller than normal bursae) with no apparent difference between infected and non-infected ones. For non-immunodepressed birds, there was no effect of GHPV infection on bursa weight: body weight ratio at 5 dpi ($p = 0.86$) or 12 dpi ($p = 0.87$). Histological examination of tissue samples collected from infected birds did not show specific lesions of the bursae, kidneys or duodenum and jejunum. Antibodies were not detected in the sera of any bird at 5 dpi, and were only detected in the sera of infected birds (groups 3 and 4) at 12 dpi (Table 5). No significant difference in mean antibody titres was observed between immunodepressed and non-immunodepressed infected birds. Results of virological analyses on bursae and sera are presented in Table 5. GHPV was never detected from tissues of non-infected birds, while it was detected from bursae and sera of infected birds at 5 and 12 dpi. GHPV was detected from the serum of one sentinel bird at 5 dpi, and from serum and bursa of two of the three sentinel birds at 12 dpi. Comparison of viral load per ml of serum between group 3 and group 4 showed an effect of dexamethasone on GHPV loads at 5 dpi (group 3 > group 4; $p = 1.3 \times 10^{-3}$). There was no difference on serum viral loads at 12 dpi ($p = 0.73$).

Discussion

It was recently shown that GHPV may infect Muscovy or mule ducks showing clinical signs of immunodepression (Pingret *et al.*, 2008). In the present work, the significance of GHPV carriage and infection in ducks was first evaluated according to a molecular analysis of “duck-GHPV” isolates, followed by experimental infection of day-old goslings. Infections of Muscovy ducklings, either immunocompetent or chemically immunodepressed, were used to investigate the pathobiology of GHPV infection in ducks.

Molecular analyses of two duck-GHPVs isolated in 2008 showed that their genomes were nearly identical to the genome described previously (Johne & Muller, 2003). The very few single nucleotide polymorphisms observed on the sequences were largely synonymous and were not host specific; indeed, none of the nucleotide changes identified on the genomes was specific of either duck or goose viruses (Table 4). This issue was carefully addressed, since in small DNA viruses such as polyomaviruses, a single amino acid change can dramatically affect viral replication and virulence (Stoll *et al.*, 1994). Experimental infection of susceptible day-old goslings by a Muscovy duck isolate or a mule duck isolate induced typical HNEG clinical signs and lesions within 5 to 16 days. Histopathology performed on kidney, duodenum, jejunum and bursa revealed identical lesions to those originally described (Lacroux *et al.*, 2004). It is therefore concluded that there is a common genotype of GHPV circulating in duck and goose flocks and that “duck-GHPV” isolates are still virulent in domestic geese. The virulence of GHPV in ducks was therefore investigated.

GHPV infection of 1-day-old and 21-day-old Muscovy ducklings did not induce any clinical or pathological changes in infected birds, by comparison with susceptible goslings of the same age (Guerin *et al.*, 2000; Palya *et al.*, 2004). However, in both experiments an active replication of the virus was demonstrated by viral detection in bursa and sera. Viral loads detected in sera of healthy infected ducklings were fully comparable to those classically detected in infected goslings displaying HNEG clinical signs (data not shown). Moreover, during the last experiment, antibodies were detected at 12 dpi, suggesting that GHPV infection induces a serological response in ducks. These data suggest that ducks can be

infected by GHPV, but this infection does not lead to any disease. Moreover, during the last experiment, it was shown that sentinel birds raised in close contact with infected ones became infected as early as 5 dpi, suggesting that GHPV was transmitted by a horizontal (likely faecal-oral) route, as has been described for geese (Guerin *et al.*, 2000). Therefore, it is concluded that GHPV can be easily transmitted within a flock.

In immunocompromised ducklings, GHPV infections neither induce clinical signs nor histopathological changes in bursae. Serological responses at 12 dpi tended to be reduced compared to normal conditions (Table 5). Serum viral loads were higher at 5 dpi in non-immunodepressed birds (Table 5), suggesting that GHPV replication could be less efficient in immunodepressed birds. These hypotheses should be confirmed by further studies. However, as immunodepressed ducklings were inoculated at 21 days of age (after the decay of maternal antibodies) and monitored for 12 days, it would be useful to confirm these results by an earlier infection and/or a longer monitoring of infected ducks.

The serological findings in the last experiment revealed the presence of GHPV antibodies in Muscovy ducklings at 7 days of age, but not at 14 days of age, thereby suggesting that these antibodies were maternally derived. Transmission of maternal GHPV antibodies was previously assessed in domestic geese (Gelfi *et al.*, 2010). Thus, it was suspected that GHPV may circulate among breeding duck farms. At the same time, we performed a field serological survey to estimate roughly the prevalence of GHPV infection in Muscovy male and Pekin female breeders. All the flocks submitted for analysis were positive for GHPV antibodies and within these flocks, all birds were found seropositive (data not shown), suggesting a widespread distribution of GHPV infection in both Pekin and Muscovy ducks. These preliminary data should be confirmed, but they suggest that GHPV infection is all but rare in domestic ducks. The epidemiological significance of this widespread infection in breeders for the viral status of their offspring is still unclear. Vertical transmission of GHPV in geese was suggested but not demonstrated by Bernáth *et al.* (2006), and should be confirmed experimentally in geese and ducks.

In these experimental infections, Muscovy duck was considered in a first attempt as a model of the different duck genotypes, since this species is considered as the more susceptible to viral infections among domestic ducks. Nevertheless, similar experiments should be performed in Pekin (*Anas platyrhynchos*) and mule ducks. Based on these experimental infections in ducklings, we conclude that GHPV is unlikely to be a significant immunodepressive agent in the duck, as initially expected (Pingret *et al.*, 2008).

Ducks are asymptomatic carriers of GHPV but show no pathological evidence of infection, and therefore should be considered as potential reservoirs of this virus. For this reason, mixed goose and duck farming systems should be now carefully reconsidered. These results highlight the role of ducks as a potential source of viral infection for other poultry species, as was described for avian influenza (Ward *et al.*, 2009) and others viral diseases (Lindh *et al.*, 2008).

The mechanisms underlying this divergence in the pathobiology of GHPV between goose and duck remain to be clarified. The virus seems to replicate very efficiently in both species, but is unable to induce any cellular damage in ducks. A harmless, non-clinical infection is the most common outcome with mammalian polyomaviruses, while polyomaviruses of birds have a particular ability to induce inflammatory diseases (Johnes & Müller, 2007). Further studies will be needed to elucidate these singular interactions between this avian polyomavirus and its hosts.

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Table 1. 5 primers designed for GHPV full sequencing.

Designation	Sequence (5' → 3')	Position	Amplicon length
VP1-1F	ggatgctgcctctaattcta	1884-1903	1232
VP1-1R	cgcagttaatcagcttaca	3097-3116	
VP2-1F	gccctactatgaaggatct	837-856	1290
VP2-1R	ggctaaagccattatcagtg	2108-2127	
SEQ2F	tgaggaagtacgggttatg	2918-2937	1308
SEQ2R	tgcttgcaatttacaaga	4207-4226	
SEQ3F	tcagctgcaaaaacaattta	4062-4081	1365
SEQ3R	tagtccaaataaggcaatg	152-171	
SEQ4F	ggttaattcctgactcaca	10-29	1271
SEQ4R	aataagcttcagttcagc	1262-1281	

Table 2. *GHPV infection of day-old goslings: experimental design, clinical signs and gross lesions.*

	Group 1	Group 2	Group 3	Group 4	Group 5
Number of goslings	5	5	5	10	10
GHPV inoculum	-	Goose Toulouse 2000	Goose Toulouse 2008	Muscovy duck Toulouse 2008	Mule duck Toulouse 2008
Infectious dose ^a	-	10 ⁸	10 ⁸	10 ⁸	10 ⁸
HNEG clinical signs	0/5	5/5	5/5	9/10	9/10
HNEG gross lesions	0/5	5/5	5/5	10/10	9/10

^a Infectious dose expressed in genome-equivalent viruses (*gev*) per bird

Table 3. GHPV infection of immunodepressed ducklings: experimental design.

	Group 1	Group 2	Group 3	Group 4	Group 5
Number of ducklings	10	10	10	10	5
Chemical immunodepression	-	Yes	-	Yes	-
Viral infection (21 days of age)	-	-	Yes	Yes	Sentinel
Infectious dose ^a	-	-	10 ⁸	10 ⁸	-
Euthanasia at day 5 dpi ^b	5	5	5	5	2
Euthanasia at day 12 dpi ^b	5	5	5	5	3

^a Infectious dose expressed in genome-equivalent viruses (*gev*) per bird.

^b Number of birds sacrificed each day post inoculation.

Table 4. *Sequence comparisons of goose and ducks GHPV genomes with the sequence of isolate Germany 2001.*

Isolate	Genome length (nucleotides)	Number of nucleotide changes (and gaps)	Amino acids changes
Toulouse Goose 2000	5252	15 (4)	ORF-X: G66A, P73S
Toulouse Goose 2008	5254	7 (2)	ORF-X: T90S, VP3: T60S
Toulouse Muscovy duck 2008	5254	8 (2)	ORF-X: T90S VP2: T169S VP3: T60S
Toulouse Mule duck 2008	5253	8 (3)	ORF-X: P60A

Table 5. *GHPV infection of immunodepressed ducklings: virological and serological results.*

Group	Viral loads				Serological titres ^a	
	Bursa		Serum		5 dpi	12 dpi
	5 dpi	12 dpi	5 dpi	12 dpi		
1	0 (0)	0 (0)	0 (0)	0 (0)	< 20	< 20
2	0 (0)	0 (0)	0 (0)	0 (0)	< 20	< 20
3	5.96 (0.73) ^b	5.24 (0.79)	8.71 (0.54)	8.23 (0.59)	< 20	700 (969)
4	3.15 (2.91)	4.60 (3.16)	7.11 (0.39)	8.36 (0.59)	< 20	230 (278)
5	0 (0)	3.23 (2.88)	3.74 (5.29)	7.31 (0.94)	< 20	< 20

^a Quantitative data.^b Mean (standard deviation).