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Age, but not anthelmintic treatment, is associated with urinary neopterin levels in semi-free ranging Barbary macaques

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Studying host parasite interactions and their implications for evolution and ecology recently received increasing attention, particularly with regard to host physiology and immunity. Here we assess variation of urinary neopterin (uNEO), a marker of cellular immune activation and immunosenescence, in response to age and anthelmintic treatment in semi-free ranging Barbary macaques (*Macaca sylvanus*). Urinary NEO levels were measured via enzyme-immunoassay from 179 urine samples of 43 individuals between 5–29 years of age. Efficiency of treatment was assessed by Mc Master flotation on repeated faecal samples, including 18 untreated individuals as control group. We used linear mixed models with age and parasite status as main effects, controlling for sex and physical condition, assessed through urinary C-Peptide-levels, with social group and ID as random factors. Urinary NEO levels significantly increased with age, suggesting that changes in aging Barbary macaque immune responses are consistent with immunosenescence described in human and nonhuman primates and can be detected via uNEO measurements. Anthelmintic treatment, however, had no influence on uNEO levels, potentially due to quick reinfections or attenuated immune responses in repeated infections. We conclude that uNEO is a potential non-invasive marker for immune function and particularly immunosenescence in wildlife.

Infectious diseases have crucial impacts on the ecology and evolution of species, influencing numerous aspects of host life history, survival and fitness^{1,2} and are considered a major cost of group living³. Infections have been studied intensely in humans^{4,5} and model species^{6,7}, and recently investigations of wildlife diseases and immune response have received increasing attention⁸. Recent advances in the field of studying senescence in wildlife⁹ demonstrate that senescence occurs in wild animals and consistently changes their physiology and immune response^{10,11}. To understand the importance of age related changes and host-pathogen interactions for evolution, studying diseases and immune responses in natural systems has become crucially important. Wildlife health monitoring has focused on different aspects of an animal's physiology that can be measured non-invasively, e.g. glucocorticoid excretion as a measure of physiological stress response^{12,13}, testosterone excretion due to its supposed, though debated, immunosuppressive effect^{14,15}, monitoring physical condition using visual criteria^{16,17}, wound healing¹⁸, and measurements of C-Peptide of insulin^{19,20} and thyroid hormones^{21,22} as markers for energetic status. Studies of age related changes currently rely mainly on invasive sampling^{10,11}, however, capturing and handling animals can be difficult or impossible and raise particular ethical concerns for endangered species or species sensitive to disturbance. Problems associated with invasive sampling make comparative work on health status challenging, so the development of non-invasively obtained measures of health- and age-related parameters are critical to this area of inquiry.

Information about immune responses of individuals with regard to certain pathogens and aging are necessary to understand the impact of infection on host fitness, as immune responses themselves are energetically costly

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or lead to energy allocation away from reproduction²³. Using blood samples is highly efficient when studying immune responses on the molecular level and have generated major insights into host-parasite interactions²⁴ and the effects of intrinsic and extrinsic factors on immune responses and reactivity^{10,25}. One current challenge in the fields of wildlife senescence and eco-immunology is the validation and establishment of suitable markers to monitor immune responses non-invasively (e.g. using faecal and urine samples) in order to better understand host parasite interactions and their implications for fitness in a wider range of systems.

Recently, advances have been made validating assays to measure markers of immune response in faecal and urine samples from nonhuman primates²⁶. One particularly promising marker is neopterin (NEO)^{26,27}, which is released from macrophages, monocytes and dendritic cells in response to interferon gamma (INF γ) stimulation²⁸ and induces T-helper 1 cell activation in the immune response against intracellular pathogens such as viruses and bacteria^{28,29}. Being also elevated in a variety of inflammatory³⁰ and non-infectious diseases^{28,31} and even under acute stress³², NEO may serve as a non-disease-specific marker of inflammation and Th1 mediated immune responses and is widely used as a diagnostic marker for disease severity and prognosis in humans in a variety of diseases²⁸. NEO is cleared unchanged via the kidneys³³ and serum and urinary NEO levels are strongly correlated in both human³⁴ and nonhuman primates^{26,35}. Supporting limited findings of an earlier study³⁵, uNEO levels increased more than 10-fold following infection with Simian immunodeficiency virus (SIV)²⁶, indicating the usefulness of uNEO level changes as a non-invasive marker of viral infection in nonhuman primates.

Apart from being a marker of acute or chronic disease associated with a Th1 immune response^{28,29}, NEO is a marker intensely studied with respect to age-related changes in the immune system. There are consistent changes in both, the innate and adaptive immune system, occurring with older age: decreased cellular immune response³⁶ and efficiency of vaccinations^{37,38}, changes in T-cell phenotypes with an increase in differentiated and simultaneous decrease in naïve T-cells^{39,40}, alterations in the innate immune system, especially changed monocyte phenotypes⁴¹ as well as reduced natural killer cell function^{40,42}, and chronic low levels of inflammation^{40,43}. Elevated NEO levels in aged individuals have not only been reported in numerous studies^{44,45}, but also correlate with several hallmark characteristics of the aged immune system in humans, amongst others altered T-cell phenotypes and changes in monocytes^{41,45}, indicating NEO as a marker of age related immune function changes.

Given the growing importance of understanding healthy aging and the processes leading to frailty and morbidity with older age in aging human societies⁴⁶, nonhuman primates have become an increasingly important model system to study immunosenescence^{47,48}, a term that summarizes the changes in the immune system of aging individuals⁴⁹. To date, most studies on immunosenescence in nonhuman primates report levels of IFN γ rather than NEO, which in most studies are elevated in older individuals^{50,51}. Since IFN γ and NEO are closely linked functionally²⁸, similar patterns are expected for NEO in aging nonhuman primates. This is supported by a recent study that reported a significant positive correlation between serum NEO levels and age in healthy macaques²⁶.

We aimed at advancing the biological validation of uNEO in nonhuman primates with regard to aging by using a cross-sectional design of young adult to aged free ranging Barbary macaques (*Macaca sylvanus*). Additionally, we aimed at assessing whether uNEO levels decrease in response to gastrointestinal (GI-) nematode infection, a group of pathogens that have been widely studied in nonhuman primates^{12,52} and other taxa^{53,54}. We capitalized on routine six-monthly anthelmintic treatment and assessed the impact of parasite clearance on uNEO levels in a cross-sectional design. Since both arms of the immune system, Th1 and Th2 are mutually inhibitory⁵⁵, high levels of NEO not only represent an efficient Th1, but also an inhibited Th2 response. As immune responses against GI-nematodes generally share a strong antibody mediated and anti-inflammatory Th2-type response⁵⁶, uNEO can potentially function as not only a direct marker of intracellular, but also an indirect marker of parasite infections. A similar relationship between GI parasite infection and Th1 responses was demonstrated in wild African buffalos (*Syncerus caffer*), where individuals with lower Th1 activity were less vulnerable to GI-nematode infections²⁴ and Th1 responses increased as a result of experimental parasite clearance. Since direct energetic costs of immune responses, including parasite defences, have been shown in several taxa^{57,58}, and clinical studies on humans have linked NEO levels to markers of energy status^{59,60}, we integrated a non-invasive measure of energy balance, urinary C-Peptides (uCP)^{20,61}, into our analyses to control for possible confounding effects of physical condition on uNEO values. We investigated the relationship between aging, GI parasite infection and uNEO levels in semi-free ranging Barbary macaques at *Affenberg Salem* in a population with a high proportion of aged individuals older than 20 years (19% of the study population). We specifically predicted increasing uNEO levels with increasing individual age in adulthood, representing changes in the immune system consistent with immunosenescence. Individuals older than 20 years were expected to have the highest uNEO levels. Additionally, we used routine anthelmintic treatment of the population to investigate the impact of GI parasite clearance on uNEO. Based on the findings of Ezenwa *et al.*^{24,54}, we predicted uNEO levels to be increased after treatment due to lack of immunomodulation by parasites.

Materials and Methods

Study site and urine sample collection. We studied two out of three freely interacting social groups of Barbary macaques in a 20 ha forested enclosure at *Affenberg Salem* in Germany. Macaques were provided with fruit, vegetables, and grains daily in the morning and had *ad libitum* access to monkey chow and water⁶². The two study groups had roughly similar age-sex compositions (group C: 20 adult females, 16 adult males, 11 immature females and 12 immature males; group H: 23 adult females, 18 adult males, 3 immature females, 8 immature males). All individuals were identifiable by physical features such as birth marks, scars, stiff fingers or stature. Urine samples from all adult individuals were collected repeatedly from individually recognized individuals six weeks prior to anthelmintic treatment to allow for collection of baseline pre-treatment data for the study population. Sampling was continued until four weeks post-treatment (group C: 2nd of June through 26th of August 2014, group H: 6th of June through the 9th of September 2015). The four weeks window was chosen to allow for

social*experimental group	H control		H treatment		C treatment	
	female	male	female	male	female	male
nr. individuals						
young (5–7 years)	3	2	2	1	2	1
prime (8–14 years)	3	3	4	3	1	1
post prime (15–19 years)	2	1	1	1	—	—
aged (>20 years)	1	3	3	3	—	2
sum individuals = 43						
nr. samples (mean \pm sd /individual)						
pre	19 (2.1 \pm 0.9)	21 (2.3 \pm 0.7)	22 (2.2 \pm 0.8)	20 (2.5 \pm 0.8)	5 (1.7 \pm 0.6)	6 (1.5 \pm 0.6)
post	21 (2.3 \pm 0.7)	20 (2.2 \pm 0.8)	19 (1.9 \pm 0.9)	18 (2.3 \pm 0.9)	3 (1 \pm 0)	5 (1.3 \pm 0.5)
sum samples = 179 (4.2 \pm 1.3)						

Table 1. Overview over sampled individuals and samples used for uNEO analysis. Distribution of study animals among social groups, age and sex classes as well as sample sizes are given for treatment and control group. Age classes were chosen for means of better visualization of data distribution, but were not used in data analyses or interpretation.

collection of several samples per individual while being able to assess the effect of reduced parasite burden. Since prepatant phases of strongyle nematodes present in the population are approximately two to three weeks⁶³ and most individuals started shedding eggs six weeks after treatment or later (unpublished data), we are confident this was actually the case. Only samples of 43 adult individuals (7 of group C, 36 of group H) with at least 1 sample pre- and post-treatment were analysed (179 samples, 4.2 ± 1.3 /individual, pre-treatment: 93 samples, 2.2 ± 0.8 /individual, post-treatment: 86 samples, 2.0 ± 0.9 /individual). The 22 sampled females were 5–29 years of age and the 21 males 6–27 years of age (Table 1). Samples were taken non-invasively using one of two methods: Urine was caught on clean plastic sheets when individuals were urinating from elevated positions (higher than 2 m) and subsequently transferred into 2 ml polypropylene cups with disposable Pasteur pipettes. We aimed at collecting samples from every individual, but had difficulties to obtain samples in 2014 as Barbary macaques are largely terrestrial and samples could not be collected when individuals urinated close to the ground. In 2015 we increased our sampling effort in order to obtain samples from more individuals. In addition to collecting urine by catching it on plastic sheets we collected urine from the ground, rocks or leaves using swabs (Salivette[®] Cortisol, Sarstedt, Nürmbrecht, Germany) when individuals urinated close to or on the ground. The latter method has recently been validated for NEO measurements in macaque and human urine⁶⁴ and allowed for a much higher sampling success. Samples contaminated with faeces were discarded as faecal contamination alters levels of uCP in macaque urine samples⁶⁵. Following collection, both salivettes and polypropylene cups were stored on ice in a thermos flask until transferring them to -20°C for long-term storage within 12 h of collection²⁷. Urine was recovered from the salivettes by centrifugation using a manually operated centrifuge⁶⁴ and was transferred to 2 ml polypropylene cups with disposable Pasteur pipettes prior to freezing. All urine samples were transported to the Endocrinology Laboratory of the German Primate Centre (Göttingen) on dry ice and stored frozen at -20°C until analysis.

Anthelmintic treatment, parasite analyses and treatment efficacy. Anthelmintic treatment was performed on the 5th of August 2014 and 17th of August 2015 as a veterinary routine measure. Individuals were fed food items containing ivermectin, a broad spectrum anthelmintic compound belonging to the macrocyclic lactone drug class, at approximately 0.4 mg/kg bodyweight, by park staff in close collaboration with the veterinarian responsible for the population. In 2015, half of the individuals of group H remained untreated as a control group ($n = 18$), which was matched for sex, age, immigration status (males) and matriline (females) to the treatment group (nine females, 5–29 years, nine males, 6–27 years of age).

Since we aimed at investigating the impact of age and parasite status on uNEO levels, confirming the efficacy of anthelmintic treatment was one prerequisite for the analyses. To do so we collected faecal samples for every individual approximately once per week for parasite analyses (pre-treatment: $n = 294$, 6.7 ± 0.9 /individual, post-treatment $n = 292$, 3.8 ± 1.4 /individual). Samples were collected as soon as possible (without disturbing the animal) after defecation noting animal ID, date, time, and observer, placed in 20 ml plastic tubes, stored on ice until being fixed with 10% formalin within 12 hours and later transported to the Institute for Parasitology of the University of Veterinary Medicine Hannover, Germany. Presence of nematode eggs was assessed using McMaster flotation, a quantitative parasitological method, after wash-out of formalin from the samples by transferring ca. 6 g to a 15 ml centrifuge tube, spinning at 2000 rpm (930 g) for 10 min, washing once with water, spinning at 2000 rpm (930 g) for 10 min and discarding the supernatant⁶⁶. For McMaster flotation, 4 g faecal matter was weighed, homogenized in saturated NaCl as flotation solution, poured through a tea strainer (mesh size 1 mm) to remove faecal particles and the sample was filled up to 60 ml with saturated NaCl. For samples with less faecal matter available, 15 ml flotation solution were used per gram and the weight of faeces used was recorded. Faecal egg counts were performed by using McMaster chambers and scanning 4 counting fields with 100- and 400-fold magnification (detection sensitivity 25 eggs/gram faeces), recording presence of parasite egg morphotypes.

Coprospectical parasite analysis revealed three nematode morphotypes, for which effectiveness of treatment differed: for strongyle nematodes, which had a pre-treatment prevalence of ~98% ($n = 42$ individuals), all treated individuals stopped shedding eggs within two days of treatment and stayed coprospectically negative for at least

three weeks. One individual excreted eggs again within three weeks and three individuals within four weeks. All other treated individuals remained coproscopically negative for at least four weeks after treatment. As strongyle eggs are difficult to differentiate by morphological characteristics, we summarized several potential genera under this morphotype, e.g. *Oesophagostomum* spp. and hookworms (*Ancylostoma* spp. and *Necator* spp.). Preliminary results from larval cultures suggest that the majority of strongyles represent *Oesophagostomum* spp., whereas only few samples contained other larval morphotypes (unpublished data). Given the short prepatent periods of the possible parasite species⁶³, this result indicates parasite clearance with rapid reinfection in few individuals. For the two other morphotypes, *Capillaria* spp. and *Trichuris* spp., prevalences were lower (*Capillaria* spp.: 37.2%, n = 16 individuals; *Trichuris* spp.: 6.9%, n = 3 individuals), but anthelmintic treatment was not efficient for all treated individuals as indicated by continued egg shedding (two study individuals and seven additional treated individuals not included in the study for lack of urine samples).

uNEO and uCP measurements. We assessed uNEO concentrations measuring duplicates for each sample using a commercial Neopterin ELISA Kit (Art. No. RE59321, IBL International GmbH, Hamburg, Germany) previously validated for the use in macaques^{26,27}. Prior to assay, urine samples were diluted 1:25 to 1:250 with assay buffer and 20 µl of the diluted urine was subsequently assayed using the manufacturer provided protocol. In this study, uNEO concentrations are provided as ng/ml by transforming the respective nmol/l values according to the manufacturer's protocol. Sensitivity of the assay was 0.34 ng/ml. Intraassay coefficients of variation (CV), determined by repeated measurement of high and low value quality controls in each assay, were 5.4% (high; n = 18) and 8.1% (low; n = 18), respectively, while interassay CVs were 6.1% (high; n = 5) and 10.5% (low; n = 5).

In addition to uNEO levels, we measured uCP concentrations as a control factor to account for individual differences in physical condition and energy balance^{19,20} as these might influence the ability of individuals to mount effective immune responses^{58,67} and influence uNEO levels^{57,59,60}. We analysed uCP measuring duplicates of each sample using a commercial C-Peptide ELISA Kit (Art. No. RE 53011, IBL International GmbH, Hamburg, Germany) previously validated for the use in macaques^{20,65}. Prior to assay, urine samples were diluted between 1:2 and 1:20 with IBL sample diluent (Art. No. RE 53017) and 100 µl of the diluted urine was then assayed according to the manufacturer's instructions. Assay sensitivity was 0.064 ng/ml. Intraassay CVs calculated from the measurement of high- and low-value quality controls (pooled human urine) were 5.2% (high; n = 18) and 8.8% (low; n = 18), respectively, while values for interassay CVs were 7.0% (high; n = 5) and 14.2% (low; n = 5).

Due to the expected sex differences in muscle mass and consequently creatinine excretion⁶⁸, we did not use creatinine but specific gravity (SG) to adjust for differences in urine volume and concentration⁶⁸. We measured SG of each sample using a digital hand-held refractometer (PAL-10S; Atago Inc., Bellevue, USA) and calculated uNEO levels and uCP levels corrected for SG by using the following formula⁶⁹: $SG - corrected\ value = raw\ value \times \frac{(SG_{mean} - 1.0)}{(SG_{sample} - 1.0)}$, with $SG_{mean} = 1.0163$.

Statistical analysis. We assessed the effect of age and anthelmintic treatment on uNEO levels using linear mixed models in R (version 3.5⁷⁰) with the package lmerTest, setting alpha levels to 0.05. After exclusion of two extremely low uNEO values that repeatedly led to extremely high residuals and prohibited log-transformation to achieve normality, both uNEO as response and uCP as predictor were log-transformed to achieve normal distribution. Rather than z-transforming age, age was rescaled by subtracting the mean age (14 years) value from individuals' values to centre the model around the mean age rather than the not meaningful value of age = 0 for the y-intercept. This was done to keep the original data structure and allow interpretation of the effect size.

Since *Trichuris* always occurred in individuals positive also for *Capillaria* both morphotypes were summarized under the factor "Cap" for statistical analysis. Based on the differences in anthelmintic treatment efficacy and the different outcomes for Cap negative (GI parasite free) vs. Cap positive (GI parasites still present), we modelled the predictors representing parasite treatment using a three-way interaction between experimental group (control vs. treatment), phase (pre- vs. post-treatment) and Cap (positive vs. negative) as main factor in the original model (Model 1), expecting smaller or no changes in uNEO levels in Cap positive vs. Cap negative treated individuals. The model contained the three-way interaction and age as main factors. As NEO has been shown to increase in elderly humans only rather than linearly across adulthood, we tested using a model with age as a quadratic term to account for the nonlinearity, however, the quadratic term was neither significant (estimate $age^2 = -0.001$, t-value = -0.498, p = 0.621) nor improving the model fit (model comparison using a likelihood ratio test with the R function anova, setting the argument test to "Chisq", Chisq = 0.390, p = 0.533), so we chose a linear fit for age. We included sex as a control variable, because rhesus macaque females showed higher levels than males in laboratory studies, though this effect was not significant²⁶. The two-way interactions of Cap, experimental group and phase were included, albeit being not interpretable given the three-way interaction, as required for model validity. The single terms Cap, experimental group, phase as well as sex and uCP were included as control factors, social group and animal ID as random factors.

Since the three-way interaction was not significant we tested if its inclusion significantly improved the model fit as a measure of model selection. Model 1 was not significantly better fitted to explain uNEO level variability than the identical model including only the two-way interaction between experimental group and phase and the single terms experimental group, phase and Cap (Chisq = 5.369, p = 0.147). Consequently, we used the less complex model for interpretation of the results (Model 2), but report results of Model 1 for reasons of transparency.

For both models, various model diagnostics were employed to confirm model validity (visual inspection of distribution of residuals, qqplots, residuals plotted against fitted values, assessing model stability using the function "glmm stability" written by Roger Mundry (MPI Evolutionary Anthropology, Leipzig), assessing leverage and dbetas for single samples and levels of the random factor ID using the package "influence.ME" and variance inflation factors using the package "car"), none of which suggested violation of model assumptions for either model. To assess the significance of the full models we compared them to reduced models excluding the age-term

all samples	mean \pm sd range	160.45 \pm 90.50 30.68–572.73	
by phase * exp. group	control	treatment	
pre	164.24 \pm 65.21 (54.55–322.89)	170.80 \pm 110.39 (59.09–518.18)	
post	157.32 \pm 70.10 (43.64–386.36)	148.03 \pm 100.69 (30.68–572.73)	
by age class			
young (5–7 years)	123.75 \pm 56.11 (30.68–295.80)	post prime (15–19 years)	166.38 \pm 88.63 (40.91–386.36)
prime (8–14 years)	151.29 \pm 81.51 (43.64–454.91)	aged (>20 years)	200.88 \pm 109.77 (71.59–572.73)

Table 2. uNEO levels (ng/ml corr. SG) in 43 adult semi-free ranging Barbary macaques at Affenberg Salem. For each category, mean \pm standard deviation and range (min to max) are given. Age classes were chosen for means of better visualization of data distribution, but were not used in data analyses or interpretation.

	estimate	std.error	confidence intervals		df	t-value	p-value
			2.5%	97.5%			
intercept	4.65	0.27	4.06	5.22	1.79	17.09	0.005
age	0.02	0.01	0.01	0.03	34.92	2.58	0.014 *
cap*exp.group*phase	0.38	0.27	−0.13	0.90	139.57	1.44	0.15
cap*exp.group	0.09	0.24	−0.35	0.55	71.88	0.40	0.69
cap*phase	−0.10	0.19	−0.47	0.27	141.28	−0.51	0.62
exp.group*phase	−0.03	0.17	−0.37	0.30	145.43	−0.21	0.83
exp.group	−0.09	0.15	−0.39	0.20	70.67	−0.55	0.58
phase	0.09	0.14	−0.18	0.35	18.10	0.62	0.54
cap	0.04	0.18	−0.30	0.38	62.09	0.22	0.82
sex	0.10	0.10	−0.08	0.29	37.00	1.08	0.29
log(uCP (ng/ml corr. SG))	0.03	0.02	−0.01	0.08	165.46	1.35	0.18

Table 3. Results of LMM (Model 1, n = 179 samples) for the effects of age and the three-way interaction of experimental group, phase and *Capillaria* infection (*Cap*) on uNEO levels. The two-way interactions between *Capillaria*, experimental group and phase were retained although uninterpretable. The single terms experimental group, phase and *Cap*, sex and uCP levels were included as control variables, social group and individual ID as random factors.

and the interaction-term between experimental group and phase while retaining all other predictors using a likelihood ratio test with the R function `anova`, setting the argument `test` to “Chisq”.

Ethical statement. This work followed the Animal Behaviour Society’s guidelines for the treatment of animals in behavioural research and teaching, and adhered to standards as defined by the European Union Council Directive 2010/63/EU on the protection of animals used for scientific purposes. Anthelmintic treatment was performed as part of the routine procedures of Affenberg Salem as defined by the European Union Council Directive 1999/22/EC and authorized by the Veterinary Office of the district office of county Lake Constance. The study was approved by the Animal Welfare Body of the German Primate Center (No. E9-16).

Results

Influence of age on uNEO in semi-free ranging Barbary macaques. This study was the first to measure uNEO in Barbary macaques. Urinary NEO levels varied between 30.7 and 572.7 ng/ml corr. SG, mean \pm sd = 160.45 \pm 90.50 ng/ml corr. SG (Table 2) and did not differ between the sexes in either of our models (Model 1: estimate \pm se = 0.11 \pm 0.10, t-value = 1.08, p = 0.29, Model 2: estimate \pm se = 0.08 \pm 0.10, t-value = 0.83, p = 0.41, see Table 3, Table 4). In accordance with our predictions, age was a significant predictor of uNEO levels, with older individuals showing higher uNEO levels (Model 2: estimate \pm se = 0.02 \pm 0.01, t-value = 2.45, p = 0.019). The size of the effect was rather small, with individuals increasing ~4% of the mean log(uNEO) in ten years (Fig. 1). However, the age effect was highly stable as indicated by the narrow confidence intervals (2.5%: 0.004, 97.5%: 0.034, see Table 4), and effect sizes are difficult to interpret due to the log-transformation of uNEO levels. Comparing the mean untransformed values between young adult (5–7 years) and aged individuals (>20 years), the difference translates into a 60% increase in uNEO levels from young to aged individuals (Table 2).

Influence of anthelmintic treatment on uNEO levels. Including the three-way interaction of Model 1 changed the estimates, but not the directions of the effects (Table 3, Table 4) and did not improve the model fit, thus only Model 2 is discussed here. For Model 2, the full model was significantly better at explaining uNEO

	estimate	std. error	confidence intervals		df	t-value	p-value
			2.5%	97.5%			
intercept	4.603	0.270	4.003	5.181	1.580	17.050	0.009
age	0.019	0.007	0.004	0.034	36.320	2.452	0.019 *
exp.group*phase	0.110	0.130	-0.144	0.364	142.520	0.846	0.399
exp.group	-0.039	0.122	-0.283	0.185	66.880	-0.381	0.752
phase	0.036	0.097	-0.152	0.224	143.410	0.369	0.713
cap	0.152	0.115	-0.064	0.372	39.650	1.324	0.193
sex	0.083	0.100	-0.109	0.227	37.400	0.827	0.413
log(uCP (ng/ml corr. SG))	0.033	0.023	-0.011	0.078	168.380	1.443	0.151

Table 4. Results of LMM (Model 2, n = 179 samples) for the effects of age and the two-way interaction of experimental group and phase on uNEO levels. Experimental group, phase, *Capillaria* infection (*Cap*), sex, and uCP levels were included as control variables, social group and individual ID as random factors.

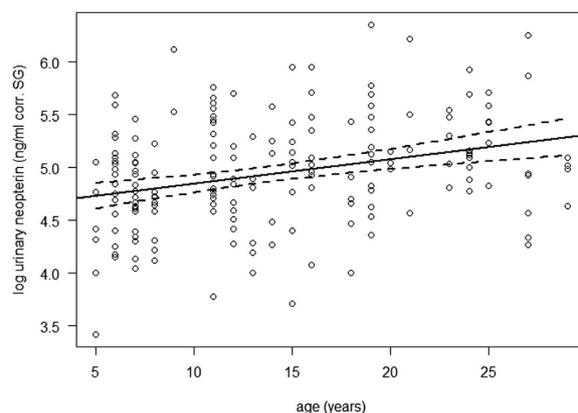


Figure 1. Effect of age on uNEO levels in 43 adult Barbary macaques. Regression line was fitted according to the estimates of Model 2 (n = 179 samples), broken lines depict 95% confidence intervals. Log-transformed uNEO-values have been plotted against the original age values to allow easier interpretation instead of age scaled to mean age used in the model (which changes the y-intercept, but neither estimates nor interpretation of the model).

variance than a reduced model excluding age and the interaction-term between experimental group and phase (Chisq = 12.18, $p = 0.032$). Neither of the control factors (sex, *Cap*, uCP levels, single terms of experimental group and phase) had a significant effect on uNEO levels (Table 4). Contrary to predictions, we found no effect of treatment (interaction between experimental group and phase) on uNEO levels (Model 2: estimate \pm se = 0.11 ± 0.13 , t-value = 0.81, $p = 0.42$, Table 4), indicating that clearance of strongyle nematodes did not lead to significant changes in uNEO levels (Fig. 2).

Discussion

In the present study, we aimed at investigating the effect of aging on uNEO in semi-free ranging Barbary macaques. Capitalizing on routine anthelmintic treatment, we assessed the potential impact of GI-nematode infection and age on uNEO levels. Anthelmintic treatment was successful for strongyle nematodes, whereas *Capillaria* and *Trichuris* infections prevailed in some individuals, which was taken into account when analysing the effect of treatment on uNEO levels. We ran linear mixed models to assess the impact of age and anthelmintic treatment on uNEO levels and found that only age showed a significant positive effect on uNEO levels, with aged individuals showing significantly higher levels than younger ones.

In both humans and the macaques in our study, NEO levels increase with age within adults, placing our results alongside findings from numerous studies investigating the link between NEO levels and aging^{44,59,71–74}. The effect of aging on NEO in nonhuman primates has not been intensively studied, but the overall processes leading to immune system alterations with age seem to be similar to those in humans^{47,48}. IFN γ , which is functionally closely linked to NEO and induces NEO release^{28,29}, rather than NEO itself, is usually measured in laboratory nonhuman primate studies. Despite some inconsistencies the majority of those studies report an increase of IFN γ with older age^{50,51}, consistent with our finding of elevated uNEO levels in aged individuals. Urinary NEO levels in our study were not linked to variation in energy balance because levels of uCP did not explain variation in uNEO levels

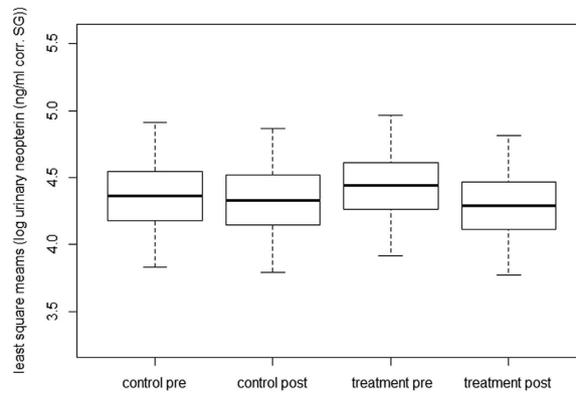


Figure 2. Effect of anthelmintic treatment on uNEO levels in 43 adult Barbary macaques. Depicted are least square means \pm se and 95% confidence intervals of least square means for Model 2 for control and treatment group pre and post anthelmintic treatment (n = 179 samples: n control pre = 40 samples, n control post = 41, n treatment pre = 53, n treatment post = 45), visualizing the modelled interaction between experimental group and phase.

over and above the effect of age. This suggests that increased uNEO levels in older individuals are not mediated by potential differences in nutritional status^{59,60} confounded with age.

Age effect size in this study was small, with a change of 4% in ten years in log-transformed uNEO levels. Elevated NEO levels are usually reported in adolescents and elderly, but not throughout adult life^{75,76}, so we tested for a non-linear effect of aging. This was not significant, making linear modelling the better approach. Apart from the possibility that there are interspecific differences in the onset of increasing NEO levels with age between humans and macaques, this result may be driven by the lack of subadult individuals in our sample. Future studies will help to shed a light on the impact of age on uNEO in nonhuman primates. However, setting our results in context with previous results from humans including healthy adults, our results are comparable with respect to the small effect in adults^{59,72,73}. Comparing the mean values of the youngest (5–7 years) and oldest (>20 years) individuals in our study group, log-transformed uNEO levels increased by 10%, which is comparable to a human study that reported a 20% increase in participants from ages 20 to 69 years⁷³.

One of the major challenges in studying immunosenescence is to disentangle changes due to older age from changes due to underlying age-related disease^{45,74}. One alternative or additional explanation for small effect size of age in our study could be the presence of many “healthy agers” more resilient to immunosenescence in our study population, since individuals more susceptible to frailty and disease are less likely to survive to old age. For this reason, very old individuals were excluded in previous studies of immunosenescence in nonhuman primates⁵⁰. Human cross-sectional studies have demonstrated that, although increases in NEO levels are a highly consistent pattern in healthy aged individuals, this development is aggravated when combined with age related disease or overall worse health: In human gerontological studies, high NEO or IFN γ have been repeatedly linked to increased morbidity (e.g. the occurrence of rheumatoid arthritis⁷⁷), frailty⁷¹ and cognitive decline⁷⁸. In one longitudinal study on elderly people, the probability of surviving until the end of the study was linked to NEO levels, with higher NEO levels relating to lower survival probability⁷⁹. Integrating NEO measurements into long-term projects and investigating the link between NEO levels, age and survival retrospectively will shed further light on whether this finding holds true in nonhuman primates.

Considering our findings, we conclude that uNEO has considerable potential as a non-invasive marker of immunosenescence in nonhuman primates. In general, immunosenescence mainly causes changes in T-cell subpopulations^{36,39}, changes in monocyte or natural killer cell populations^{41,42}, chronic, low level inflammation⁴³ and overall poorer immune system performance in response to challenges, e.g. vaccination^{37,80}, none of which can be investigated non-invasively to date. However, these immune system changes correlate with increased NEO levels⁴¹. Additionally, adverse health factors, such as HIV infection⁴⁰, inflammatory processes and prolonged physiological stress^{36,43} have been shown to induce changes in the immune system that mirror immunosenescence. These are assumed to accelerate and intensify age related changes in the immune system while being linked to elevated NEO levels^{40,41}. Thus, uNEO could serve as an immunosenescence marker, as well as a marker for overall health in wildlife, with chronically elevated uNEO levels indicative of worse health and lower expected survival time in aged individuals. Despite intensive efforts to define consistent changes throughout life history in humans^{74,75,76}, defining markers and threshold values for healthy aging remains challenging. NEO offers a valuable addition to studies of health and aging in wildlife, particularly if combined with other health-linked parameters, such as glucocorticoid hormone levels, visual frailty, wound healing patterns, or energy status, when addressing question of eco-immunology, health and fitness under natural conditions.

The picture concerning the impact of parasites on uNEO levels is less clear, as we found no changes of uNEO levels in response to anthelmintic treatment. Based on the general pattern of immune responses against GI parasites and previous findings in buffaloes^{24,54}, where parasite clearance increased Th1-activity in treated individuals^{24,54}, we predicted an increase in uNEO levels in response to anthelmintic treatment. Since NEO is connected to Th1 responses^{28,81}, inflammation^{28,71}, acute stress^{32,71} and severity of various infectious and non-infectious

diseases in humans²⁸, the lack of evidence for an impact of parasite removal on uNEO levels in this study is surprising. However, several factors could lead to the effects of treatment being masked or attenuated in our study: First, the anthelmintic compound used for treatment, ivermectin, has been reported to improve immune function in studies on various species, e.g. humans, sheep, pigs, rabbits and rats^{82–85}. However most studies used long-term treatment rather than single doses⁸², higher than therapeutic dosages⁸³, ivermectin treatment in absence of parasites, or experimental infections with a single parasite species^{85,86} and reported only transient effects of ivermectin⁸³. Therefore, we conclude that the impact of the ivermectin treatment on uNEO levels in our study is probably negligible.

Second, different parasite species elicit distinct and highly specific immune responses. Both hookworms and *Oesophagostomum* spp. have been demonstrated to elicit mixed Th1/Th2 type responses⁸⁷ and *Oesophagostomum* spp. tend to cause chronic rather than transient infections⁸⁸, potentially due to a Th1-directed shift and lack of protective immunity⁸⁷. Likewise, *Trichuris* infections are usually transient⁸⁸, but can become chronic if animals fail to mount efficient Th2-responses⁸⁹. These shifts towards a more Th1 prone response are not only based on host genetics^{24,89}, but can also be induced by the parasites themselves in an arms-race with their host. Evidence is mounting that GI parasites actively manipulate the host immune system via excretory substances⁹⁰, e.g. both human hookworms and murine *Trichuris* produce substances that mimic IFN γ or induce its release^{91,92}. Consequently, both immunomodulation by the parasites and parasite clearance can cause heightened Th1 responses^{24,92}, leading to unchanged uNEO levels after treatment.

Third, several individuals in our study became GI parasite positive again quickly after treatment, as indicated by shedding of parasite eggs within four weeks after treatment. This could explain the difference between our lack of finding and the results of studies on ungulates where individuals treated with a compound bolus were parasite free for up to 24 months^{24,54}. We can expect tissue damage due to either wandering hookworm larvae^{91,93} or encystation of *Oesophagostomum* larvae in the gut mucosa⁶³ in freshly infected individuals. Early immune responses are expected against parasitic antigens as well as tissue damage. Consequently, it is possible that changes in uNEO levels occur on a short timescale which we were unable to capture with our sampling regime. Similarly, the effects of SIV infection in experimentally infected rhesus and longtailed macaques were pronounced, but transient, with clearly elevated uNEO levels between 10–20 days after infection and subsequently moderately elevated levels²⁶, with the changes expected in our experiment being smaller than those in an infection with SIV.

Fourth and most importantly, individuals in the study population are treated regularly and are thus infected repeatedly on a regular basis. Laboratory studies on immune-parasite interactions are often on first infections, which usually elicit strong immune responses⁹⁴. Subsequent infections often lead to less pronounced immune response patterns^{95,96}. Even in infections such as malaria in which NEO levels are expected to rise markedly, repeated infections have been demonstrated to attenuate the NEO response⁹⁷. Given that for most host individuals, parasite burdens will be low while only a few show high parasite burdens⁹⁸ and natural infections with GI parasites usually occur via low-dose trickle infections⁹⁶, changes in the infection status may not necessarily lead to pronounced changes in the immune system balance of most hosts. Additionally, infections early in life, which are to be expected in our study population, have been shown to induce long lasting shifts of the immune system towards Th2 responses that are not influenced by repeated anthelmintic treatment⁹⁹, a scenario that might well mimic the situation of our study population. Further studies using more frequent sampling and focussing on first reinfections in young individuals might help to evaluate whether NEO can be used as an indirect marker of GI parasites in nonhuman primates.

Conclusion

In accordance with previous studies on human and nonhuman primates, aged Barbary macaques of our study groups showed higher uNEO levels compared to younger adult individuals. This leads us to conclude that semi-free ranging Barbary macaques, presumably faced with a more natural range of pathogens than are laboratory nonhuman primates, show signs of immunosenescence which can be measured by uNEO levels. For these reasons, we argue that uNEO is a potential marker for studies on wildlife health, particularly for studies on the effects of aging, despite the lack of effect of parasite clearance on uNEO in our study subjects. We suggest future studies linking longitudinal data on uNEO levels with data on individuals' survival and reproductive success. Including uNEO measurements might prove a valuable contribution to studies of aging, decreased immune function, morbidity and factors determining longevity and survival in wildlife.

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Author Contributions

J.O., O.S. and N.M. designed the study. N.M. collected the data. N.M. and C.S. performed the parasite analysis. M.H. performed the uNEO and uCP analysis. N.M. took the lead in paper writing and created the figures, all other authors contributed to the writing. All authors gave final approval for publication.

Additional Information

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