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Reduced gene expression of intestinal α -defensins predicts diarrhea in a cohort of African adults

Paul Kelly^{1,2,3}, Mona Bajaj-Elliott^{1,4}, Max Katubulushi², Isaac Zulu², Richard Poulson⁴, Roger A Feldman¹, Charles L Bevins⁵, and Winnie Dhaliwal¹

¹ Centre for Adult and Paediatric Gastroenterology, Institute of Cell and Molecular Science, Barts & The London School of Medicine, London, UK

² University of Zambia School of Medicine, Lusaka, Zambia

³ Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, UK

⁴ Infectious Diseases and Microbiology, Institute of Child Health, London, UK

⁵ Cancer Research UK, Lincoln's Inn, London, UK

⁶ Department of Medical Microbiology and Immunology, University of California, Davis, USA

Abstract

Background & Aims—The Paneth cell α -defensins HD5 and HD6 contribute to the antimicrobial barrier against intestinal infection. We have previously demonstrated that both HD5 and HD6 mRNA were reduced in adults living in urban Zambia compared to adults living in London. The aim of this study was to determine if α -defensin expression in Zambian adults is related to susceptibility to diarrhea during 3 years of follow-up.

Methods—We analyzed intestinal biopsies from a longitudinal cohort study in 83 Zambian adults using quantitative reverse transcription polymerase chain reaction (RT-PCR), Western blotting, immunohistochemistry and *in situ* hybridization, and we measured diarrhea incidence.

Results—HD5 and HD6 mRNA in Paneth cells varied between individuals, over time and seasonally, and was strongly correlated with mucosal architecture. Gene expression was restricted to Paneth cells. The median (interquartile range, IQR) HD5 mRNA was 6.0 (5.6-6.7) \log_{10} transcripts/ μ g total RNA in 18 participants who experienced diarrhea in two months after the biopsy was taken, compared to 6.8 (6.2-7.3) in 94 who did not ($P=0.006$), and a similar observation was made for HD6.

Conclusions—These data indicate that intestinal α -defensin expression is dynamic and seasonal, and suggest that susceptibility to intestinal infection is related to defensin expression.

Corresponding author: Dr Paul Kelly, Centre for Adult and Paediatric Gastroenterology, Institute of Cell and Molecular Science, Barts & The London School of Medicine, Queen Mary, University of London, Turner Street, London E1 2AD, UK Tel: +44 20 7882 7191; Fax: +44 20 7882 7192; email: m.p.kelly@qmul.ac.uk.

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(4) Corresponding author: Dr Paul Kelly, Tropical Gastroenterology & Nutrition group, Department of Medicine, University of Zambia School of Medicine, University Teaching Hospital, PO Box 531X, Ridgeway, Lusaka, Zambia, Tel and Fax: 00260 1 252269, email: guts@coppernet.zm

Keywords

defensin; diarrhea; Africa; innate immunity; Paneth cell; incidence; enteropathy; HIV

Introduction

Intestinal infectious disease is a major contributor to morbidity and mortality in tropical countries, estimated to have caused 1.5 million deaths in children in developing countries in the year 2002¹. Although the death rate from acute diarrheal disease in children has fallen due to the widespread use of oral rehydration therapy, the more refractory problem of the persistent diarrhea-malnutrition syndrome remains a public health problem². A similar syndrome has emerged in adults with HIV-related immunosuppression, and both adults and children with these disorders have high mortality rates^{3,4}. In AIDS, in primary malnutrition, and during immunosuppressive therapy, there is a failure of cell mediated immunity, so there is an urgent need to elucidate pathways of intestinal host defense. An improved understanding of innate immunity may lead to new therapeutic avenues for persistent intestinal infections.

Antimicrobial peptides^{5,6} secreted into the intestine may constitute an important barrier to colonization of enterocytes (i.e. infection) and to the translocation of bacteria from the lumen into blood and lymph. Paneth cells are specialized intestinal epithelial cells which contain a rich armamentarium of antimicrobial peptides and proteins. In addition, cells of other lineages also secrete molecules with antimicrobial properties. The intestinal α -defensins, human defensin (HD)⁵ and HD6, are small cationic peptides synthesized by Paneth cells^{7,8} which probably make a major contribution to intestinal defence^{9,10,11}. Mice with a deletion of the matrilysin gene cannot process α -defensins to their active form and are more susceptible to colonization with *Salmonella* spp.¹². Also, transgenic mice which express HD5 are protected from lethal challenge with *Salmonella typhimurium*¹³ indicating that HD5 can function as an antimicrobial molecule *in vivo*.

We have previously observed that adults living in a crowded township in Lusaka, Zambia, had lower levels of mRNA of both HD5 and HD6 than adults living in London¹⁴, yielding approximately 10-fold less specific mRNA per μ g total RNA. This is surprising as it might be expected that increased expression of antimicrobial peptides might confer a survival advantage in tropical populations where exposure to intestinal pathogens is frequent and intense. It has been known for many years that healthy members of tropical populations have a background environmental enteropathy. This 'tropical enteropathy'¹⁵ appears to be a consequence of high exposure to enteropathogens as it is largely reversible in visitors to the tropics¹⁶, it is more closely related to economic circumstances than to climate¹⁷, and it shows seasonal variation¹⁸. Tropical enteropathy is characterized by reduced villous height, increased crypt depth, and T cell activation when compared to populations living in temperate climates, both in adults¹⁹ and children²⁰. This pattern of crypt hyperplastic enteropathy also characterizes many infective states^{21,22}, but within our Zambian population villous height and crypt depth were positively correlated¹⁸ as occurs during starvation²³. We studied the pattern of Paneth cell defensin expression in adults in this tropical population and in relation to the individual risk of diarrhea during two years of a longitudinal cohort study.

Methods

Study groups and tissue collection

We studied 83 Zambian adults, drawn at random from a cohort study, fully representative of the population of a crowded shanty compound in Lusaka in which we have previously analyzed small intestinal architecture and function^{18,24}. HIV seroprevalence in Lusaka is relatively stable at 25-30%²⁵ so HIV status was established in those participants willing to undergo testing. Jejunal biopsies were obtained by enteroscopy approximately annually over this time period, and used for quantification of HD5 and HD6 mRNA¹⁴ and for localization of mRNA and peptide. Morphometry was carried out as previously described, including villous height, crypt depth, villous width, epithelial surface area, and villous compartment volume, but only the first two measurements were made on biopsies taken after baseline¹⁸. Paneth cells were counted as previously described²⁶. Biopsies for morphometric analysis, immunohistochemistry and *in situ* hybridization were collected into formal-saline; biopsies for RT-PCR and peptide analysis were immediately snap-frozen in liquid nitrogen, stored at -80°C , and analyzed within 6 months. Approval for these studies was obtained from the research ethics committees of both the University of Zambia and the London School of Hygiene and Tropical Medicine.

Competitive RT-PCR for HD5 and HD6

We have previously described a quantitative assay for HD5 and HD6 mRNA¹⁴. Briefly, biopsies were treated with Trizol (Invitrogen, Paisley, UK) for RNA extraction, treated with DNase (Promega, UK), and co-reverse transcribed with known quantities of a standard synthetic RNA prior to PCR amplification. The threshold of detection was determined to be 10^4 transcripts per μg total RNA.

Immunohistochemistry and in situ hybridization

Immunohistochemistry was used to define expression of HD5 as previously described²⁶. No antibody to HD6 was available. HD5 and HD6 mRNA distribution was defined using *in situ* hybridisation²⁶.

Peptide isoforms

To determine if there was variation in the stored forms of HD5, tissue extracts of cationic peptides were analyzed by acid-urea gel electrophoresis, followed by Western blotting as previously described²⁷. Recombinant HD5 and pro-HD5 peptides were used as markers of migration, and a control antibody from animals injected with vehicle only was used as a negative control.

Studies of diarrhea incidence

Participants in the Lusaka cohort study were interviewed every 2 weeks to ascertain whether they had experienced diarrhea in the previous 2 weeks. Participants who had experienced diarrhea were not invited for endoscopy until one month had elapsed, but otherwise dates of appointment were allocated randomly at any time of the year (except for July or August) in order to detect seasonal variation.

Data analysis

HD5 and HD6 mRNA content was expressed as \log_{10} transcripts per μg total RNA. The levels were not normally distributed so results are presented as median and interquartile range, and in statistical comparisons non-parametric statistical tests, the Kruskal-Wallis test, Wilcoxon's matched-pair rank sum test, and Spearman's rank correlation coefficient, were used. Where the result of the RT-PCR was below threshold (i.e. below 10^4 transcripts/ μg)

the result was designated 4.0 for the purposes of analysis, which would not affect non-parametric test results. Seasonality was assessed by presenting villous height and mRNA in two-month periods from all years of data collection grouped together. Data on diarrhea incidence was used to estimate incidence as a dichotomous (yes/no) variable for the two months before or after the date of the biopsy. Analysis was performed using STATA version 8.0 (Stata Corp, College Station, Texas).

Results

Dynamics of α -defensin expression in Zambian adults

We analyzed biopsies taken from 83 participants in the first year of the study then analyzed biopsies taken from those individuals who remained under follow up; in the second year 46 of these were still under follow up and in the final year 40 were still included, so 169 biopsies altogether were available for analysis. Characteristics of the study participants at baseline are shown in Table 1 together with HIV status (where known), CD4 counts, morphometry of the biopsies, and defensin mRNA.

The number of transcripts in initial biopsies from 83 different individuals varied from 4 (i.e. threshold) to 8 \log_{10} transcripts / μg total RNA, and a similar range was seen in every year of the study. This range of variation was not explained by inter-experimental variation, and exceeded the variation seen in triplicate biopsies taken from the same individual¹⁴. The correlation between HD5 and HD6 mRNA was strong ($\rho=0.88$; $p<0.001$). No overall differences were seen in HD5 and HD6 mRNA between HIV infected and uninfected adults (Table 1). No correlation was observed between mRNA content and CD4 count in HIV-infected participants. There was also substantial longitudinal variation between the values obtained from each individual over the two years of the study (Figure 1). In the group as a whole the longitudinal variation displayed a seasonal effect (Table 2).

This variation in mRNA levels was not due to changes in the cell types expressing HD5 and HD6 as assessed by *in situ* hybridization and immunohistochemistry. HD5 and HD6 were almost exclusively expressed by Paneth cells (Figure 2) with only one or two cells higher up the crypt staining by either technique in all the 60 sections examined, and Paneth cell numbers varied little or not at all. As HD5 is stored as propeptide²⁷ we looked for evidence for variation in quantity of stored propeptide or in post-translational processing accompanying the changes in mRNA quantity. Western blots of cationic peptide extracts of these biopsies showed the profile of peptide isoforms previously observed in human intestinal tissue²⁷, including propeptide and O-glycan linked propeptide (Figure 3). Very little variation was observed in either the quantity or the migration pattern of HD5 propeptide isoforms stored in Paneth cell granules (Figure 3). As no antibody for HD6 was available, peptide analyses were only performed on HD5.

HD5 and HD6 mRNA varied with changes in mucosal architecture

In HIV seronegative adults, HD5 and HD6 mRNA expression at baseline were both inversely correlated with villous height, epithelial surface area and villous compartment volume. No correlation was seen with crypt depth (Table 3). Over 2 years of follow up, the changes in defensin mRNA expression showed strong inverse correlations with the changes in villous height and significant correlations were also seen with the observed changes in crypt depth (Table 3). The seasonal variation in defensin mRNA was inversely correlated with the seasonal variation in villous height (Table 2). In HIV seropositive individuals no correlations between defensin mRNA and determinants of mucosal architecture were observed.

Inverse correlation between defensin mRNA and incidence of diarrhea

Defensin mRNA was compared in biopsies from participants who went on to experience diarrhea in the two months after the biopsies were taken and those who did not. Median (IQR) HD5 mRNA was 6.0 (5.6-6.7) in 18 participants who experienced diarrhea compared to 6.8 (6.2-7.3) in 94 who did not ($P=0.006$). Similarly, HD6 mRNA was 6.0 (5.6-7.3) in 20 who experienced diarrhea compared to 6.6 (6.2-7.5) in 99 who did not ($P=0.04$).

Discussion

Innate immunity in the small intestine is likely to make a major contribution to host defense, being an initial barrier to the colonization or translocation of potential pathogens. Experiments in animals suggest that defensins have a significant role *in vivo* in intestinal defense, although recent *in vitro* data suggest that HD6 is not antimicrobial against a limited number of bacterial species of the intestinal microbiota²⁸ and the authors speculate that HD6 may have activity against viral or other pathogens. There are few data from studies in humans, and confirmation of such a role would be of great interest. We found that small intestinal α -defensin expression is constitutive, in as much as it is always detectable. However, mRNA varied substantially between individuals, over time, and in a seasonal pattern. mRNA varied reciprocally over time with both villous height and crypt depth, which were positively correlated with each other¹⁸.

We also found that reduced gene expression was associated with an increased risk of diarrhea. There are two possible explanations for this. The first is that defensins make such an important contribution to intestinal defense that a 10-fold reduction in mRNA could significantly increase susceptibility to infection and therefore diarrhea. This is plausible as mRNA predicted peptide turnover in experiments in an *ex vivo* model (W. Dhaliwal, unpublished observations). The second is that increased exposure to tropical microbiota could simultaneously down-regulate defensin mRNA and increase diarrhea incidence. This hypothesis is supported by experiments which suggest that pathogens or their components can suppress expression of antimicrobial molecules^{29,30}. However, our study design required that participants should have been free of diarrhea for the month prior to the date of biopsy, so we favor the first explanation, although the latter cannot be completely ruled out.

The strong correlations over time between defensin mRNA and mucosal architecture deserve further exploration. The very strong correlation between HD5 and HD6 made it important to establish whether the changes in defensin transcripts might be explained by changes in Paneth cell number or distribution, or changes in the lineage of cells expressing α -defensin genes, but no such changes were found. It is well known that T cell activation induces mucosal remodeling, with reduced villous height and increased crypt depth. This may explain the differences between temperate and tropical populations, in which T cell activation has been suggested to play a part¹⁹. However, it cannot explain the mucosal remodeling within this tropical population in which villous height and crypt depth were positively correlated¹⁸. We suspect that nutritional factors may play a part as starvation is the only context in which such a positive correlation has so far been observed. Although to our surprise there was no difference in mRNA quantity between HIV-infected and HIV-uninfected people, the correlation between mRNA and mucosal architecture was lost in HIV infected adults. This suggests that in HIV infection there is some dysregulation of α -defensin transcriptional control.

Diarrhea incidence was greater in participants who had reduced defensin mRNA. We can only find significance in this observation if we are reasonably confident that exposure to potential pathogens would be similar from one individual to another. This is highly probable in this cohort study, because all participants were drawn from one small subsection of one

'compound' in peri-urban Lusaka. This environment is characterized by overcrowding and poor sanitary facilities. Water and food outlets are few in number and invariably hygiene is poor. A majority of adults living in this area at baseline participated in our study, and we believe that exposure, although not uniform, was likely to be quite consistent. We can be confident that the reduced defensin mRNA in patients who went on to experience diarrhea was not confounded by HIV status, which although predictive of diarrhea incidence (data not shown), was not related to defensin mRNA as noted above. Future studies will be needed in other populations to confirm the association between reduced defensin expression and increased risk of diarrhea. It will also be necessary to establish whether this effect is through increased frequency of infection (by which we mean colonization), increased intensity of infection, or a permissive effect on virulence factor expression by pathogens.

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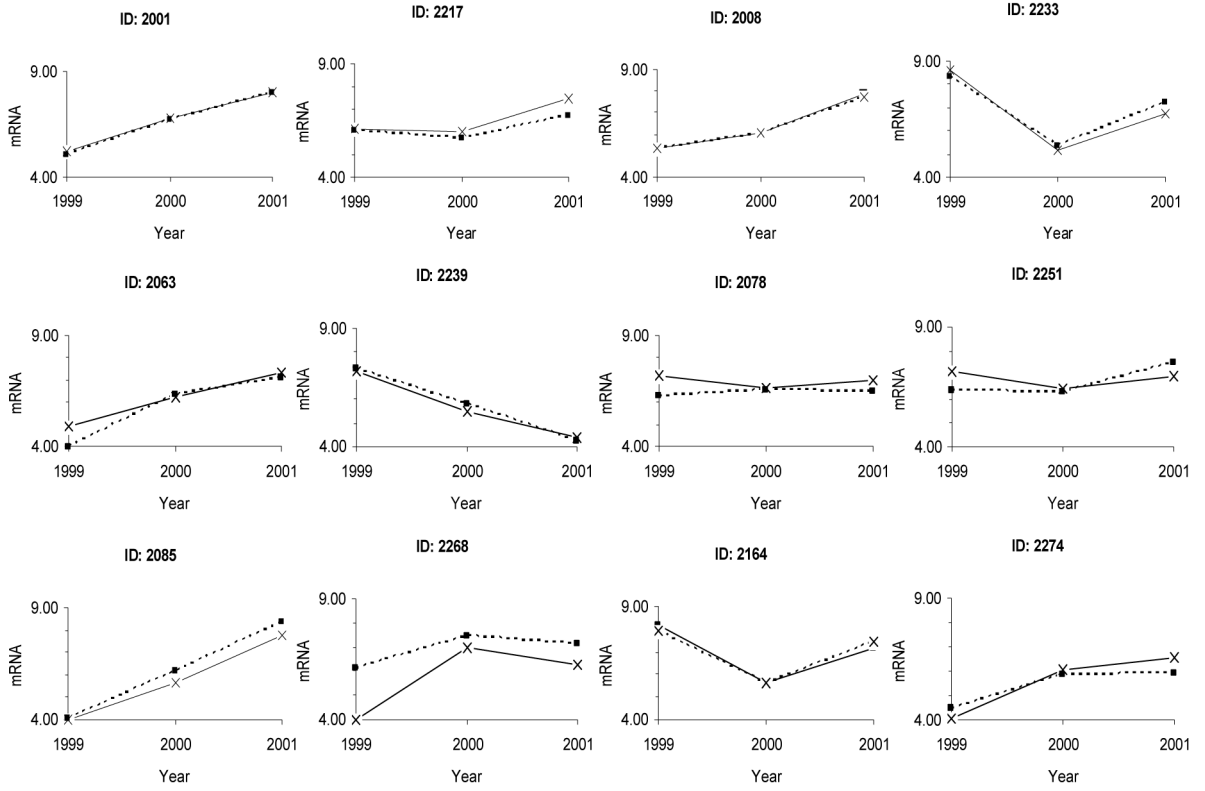


Figure 1. Longitudinal changes in HD5 (solid line) and HD6 (dashed line) mRNA at 3 time points over 2 years. Data from twelve participants from the Lusaka population have been selected to demonstrate the range of variation seen. mRNA is shown as \log_{10} transcripts/ μg total RNA.

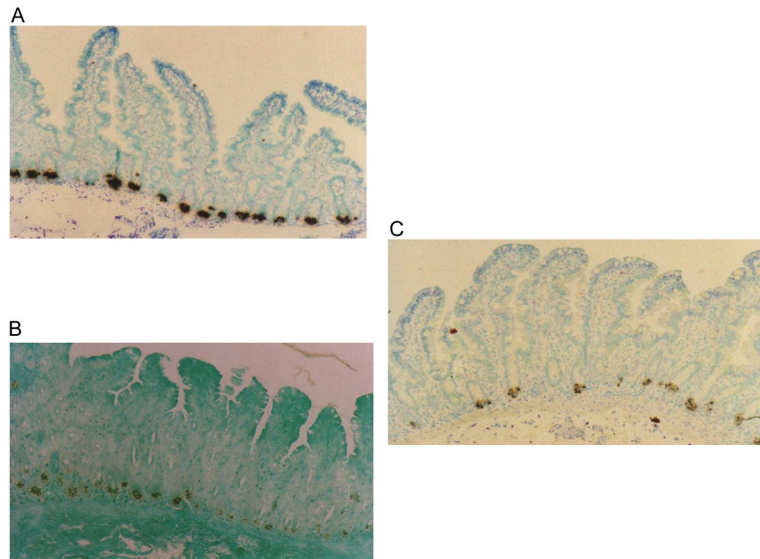


Figure 2. **A** *In situ* hybridization using antisense riboprobe to HD5. **B** Immunostaining using monoclonal anti-HD5 antibody. **C** *In situ* hybridization using antisense riboprobe to HD6. Labelling of mRNA and peptide was almost exclusively localized to the Paneth cell compartment.

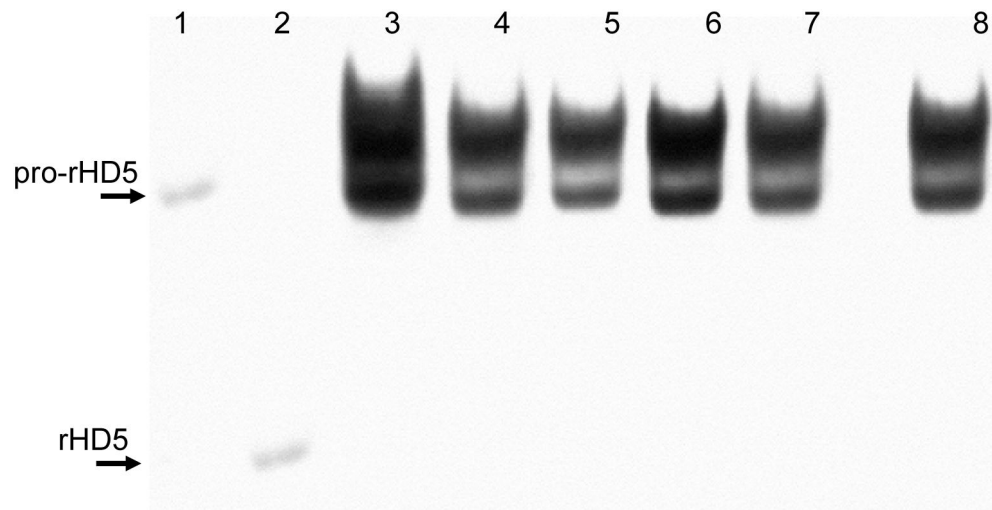


Figure 3.

Western blot of tissue extracts of jejunal biopsies probed with anti-HD5 antibody. Gel electrophoresis was performed by acid-urea polyacrylamide gel electrophoresis in which peptides are separated by both size and charge. Recombinant peptides are therefore used as markers of migration. Lane 1, recombinant pro-HD5 (20-94); lane 2, recombinant HD5; lanes 3-7, individual biopsies; lane 8, control ileal tissue from healthy patient. The quantity and isoform distribution of propeptide shows little variation. The diffuse, slow migrating band above the pro-HD5 control corresponds to an O-linked glycan modification of the HD5 (20-94) propeptide (C. L. Bevins, unpublished data).

Table 1

Demographic and clinical characteristics of Lusaka study participants at baseline

	All	HIV seronegative	HIV seropositive	<i>P</i>
n	83	47	31	
Sex (M:F)	30:53	19:28	10:21	0.48
Age (years)	32 (27-40)	39 (29-45)	29 (27-35)	0.006
VH (μm)	256 (222-299)	261 (217-310)	253 (225-279)	0.39
CD (μm)	153 (135-173)	143 (131-161)	169 (152-186)	0.001
CD4 (cells/ μL)	611 (389-800)	722 (620-855)	333 (194-471)	0.0001
CD8 (cells/ μL)	625 (391-774)	506 (383-643)	792 (589-1337)	0.0005
HD5 (\log_{10} transcripts/ μg)	5.6 (4.6-6.7)	5.9 (4.9-6.9)	5.2 (4.0-6.2)	0.31
HD6 (\log_{10} transcripts/ μg)	5.8 (5.0-6.5)	6.0 (5.2-6.6)	5.7 (4.6-6.2)	0.22

83 participants were included in the study but HIV tests were performed only on 78. Values shown are given as median (interquartile range), and the *P* value given was derived from a Kruskal-Wallis test of the difference between HIV seropositives and HIV seronegatives. (VH, villous height; CD, crypt depth).

Table 2

Seasonal variation in defensin expression

Months	n	HD5*	HD6*	VH	CD
Jan, Feb	7	8.1 (8.0-8.2)	8.4 (7.9-8.4)	202 (176-215)	157 (130-191)
Mar, Apr	35	6.2 (5.3-6.9)	6.3 (5.6-6.7)	258 (234-304)	164 (140-193)
May, Jun	70	5.9 (5.0-7.2)	6.2 (5.2-7.4)	254 (231-302)	156 (132-172)
Sep, Oct	45	6.5 (5.6-7.0)	6.4 (5.6-7.0)	230 (218-258)	152 (138-168)
Nov, Dec	12	6.2 (4.0-6.9)	6.2 (5.7-6.7)	217 (194-262)	149 (134-173)
<i>P</i>		0.001	0.002	0.0001	0.59

* Values given are median (interquartile range) of the mRNA (log10 transcripts/ μ g total RNA) of all the biopsies taken during those months during the entire 2 year study period. Villous height (VH) and crypt depth (CD) are expressed in μ m. *P* values refer to results of the Kruskal-Wallis test across all groups. No sampling was performed in July or August. Rainfall occurs during November – March with peak rainfall in January when mRNA was at its highest.

Table 3

Correlation between mucosal architecture and defensin mRNA in HIV seronegative participants, at baseline and over 2 years of follow up

Architectural variable	ρ	<i>P</i>	ρ	<i>P</i>
<i>Baseline parameters</i>		<i>HD5</i>		<i>HD6</i>
Villous height	-0.39	0.01	-0.36	0.01
Crypt depth	-0.21	ns	-0.15	ns
Epithelial surface area	-0.42	0.006	-0.38	0.01
Villous compartment volume	-0.39	0.01	-0.43	0.004
Villous width	0.32	0.04	0.21	ns
<i>Change over 2 years</i>				
Villous height	-0.71	0.005	-0.64	0.01
Crypt depth	-0.54	0.04	-0.53	0.04

Coefficients shown are Spearman's rank correlation coefficients (ρ) for biopsies from 42 participants at baseline and for 15 participants followed up for 2 years. The correlations in HIV seropositive participants were weak and statistically insignificant. (ns, not statistically significant).