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Keratinization of the Adult Male Foreskin and Implications for Male Circumcision

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Abstract

Objective—The theory that a more thinly keratinized inner foreskin leads to increased HIV-1 susceptibility has been based on relatively little published data. We sought to quantify the keratin thicknesses of the inner and outer foreskin to determine the plausibility of this hypothesis.

Design—We took repeated measurements of the keratin layer of 16 adult male foreskins to determine if differences existed between the inner and outer foreskin.

Methods—Adult foreskins were collected from consenting donors undergoing elective male circumcision for unknown medical indications in Chicago, IL. Specimens were processed, sectioned and stained for keratin using anti-filaggrin fluorescent antibodies. Slides stained with H&E were used as controls to compare to previously published studies using this method. Keratin layers were measured in a standardized fashion for each specimen.

Results—Comparing our fluorescence-based analysis to previously published immunohistochemical methods revealed that our method was highly accurate for measuring foreskin keratin thickness. There was significant heterogeneity in the keratin thickness of the inner and outer aspects of the male foreskin within and between the different donors. There was no significant difference between the inner and outer foreskin keratin thickness (25.37 ± 12.51 and $20.54 \pm 12.51 \mu\text{m}$ respectively, $p=0.451$).

Conclusions—We found no difference between the keratinization of the inner and outer aspects of the adult male foreskin. Keratin layers alone are unlikely to explain why uncircumcised men are at higher risk for HIV infection.

Keywords

HIV; Transmission; Male Circumcision; Foreskin; Keratin

Introduction

In recent years, clinical trials in Africa demonstrated that male circumcision provides up to a 60% risk reduction in HIV acquisition [1–3]. The scientific rationale(s) behind this protective effect has been highly speculative to date. One theory proposes that more potential HIV-1 target cells in the inner aspect of the male foreskin accounts for higher infection rates in uncircumcised men [1,2]. However, no differences were noted between target cells in the inner and outer

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foreskin in a study that pre-dated the African circumcision trials [3]. Our group has also shown that the glans penile epithelia actually contains more target cells than foreskin epithelia, further refuting the theory that target cells in the foreskin alone explain how uncircumcised men are more susceptible to HIV infection [4].

Another theory has been that the inner foreskin has a thinner keratin barrier (cornified layer) than either the outer foreskin or penis, making it more vulnerable to infectious pathogens [2, 5]. A thinner barrier at the surface could decrease the distance between viruses and underlying target cells, thereby facilitating acquisition. It could also be more susceptible to environmental processes that compromise the integrity of the epithelial barrier. There have been only three reports comparing foreskin keratin thicknesses to date [2,5,6]. Two studies found the outer foreskin to be more thickly keratinized than the inner foreskin, though one did not show quantifiable data and the other used an arbitrary scale. Qin et al. measured the keratin thickness and found the opposite result of what the first two groups had reported—that inner foreskin keratin was actually thicker than that of the outer foreskin. These conflicting reports indicated that additional analysis was required to determine the importance of the keratin layer in HIV transmission.

In this study, we defined keratinization in the human adult male foreskin utilizing multiple staining methods. We also sought to determine if differences existed between the keratin layer thickness of the inner and outer foreskin. This analysis may prove important in understanding how circumcision status can influence female-to-male HIV transmission.

Methods

Specimen Collection and Processing

Adult male foreskin specimens were collected from consenting donors undergoing elective circumcision at Northwestern Memorial and Rush Presbyterian Hospitals in Chicago, IL. Prior to this study, approvals from the Northwestern and Rush Institutional Review Boards were obtained. In order to protect patient confidentiality, no identifying information was obtained or recorded. We also did not obtain information on pre-existing medical conditions, though all specimens with grossly visible lesions or ulcers were excluded. The specimens were immediately processed after surgical excision from the donors and washed once in 1X phosphate buffered solution (PBS, HyClone). Each specimen was separated into the inner and outer aspects of the foreskin using the following criteria: 1) darker pigmentation of outer foreskin as compared to inner foreskin 2) smoother surface of the inner foreskin 3) smaller surface area of the inner foreskin. Specimens that did not have clear inner and outer aspects were not used in our analysis. Small pieces of inner and outer foreskin from each specimen were frozen in optimal cutting temperature medium (OCT, Sakura) or fixed in 3.7% formaldehyde (Polysciences) and paraffin-embedded. ~10 µm cryosections were obtained on glass slides for staining. An independent party blinded all slides prior to immunofluorescence staining and analysis (i.e., the party responsible for staining and analyzing the slides was unaware of the type of tissue they had on the slides).

Immunofluorescence

Frozen tissue sections were fixed in 0.1M PIPES buffer, pH 6.8 and 3.7% formaldehyde (Polysciences). Sections were blocked with 10% normal donkey serum (Jackson ImmunoResearch Laboratories, Inc.), 0.1% Triton X-100 and 0.1% Sodium Azide (Sigma-Aldrich). Monoclonal mouse anti-human α -filaggrin antibody (Santa Cruz Biotechnologies) was used as a primary antibody (a ~35kDa protein that is produced by terminally differentiating squamous epithelial cells); rhodamine red-conjugated donkey anti-mouse antibody (Jackson ImmunoResearch) was used as a secondary antibody for staining. Slides were washed in PBS

at 4°C in between antibody incubations. DAPI (4',6-diamidino-2-phenylindole; Invitrogen) and Wheat Germ Agglutinin (WGA, Invitrogen) was used to visualize nuclei and cellular membranes (by binding to cellular surface carbohydrates), respectively. Hematoxylin and eosin (H&E) staining was performed by the Northwestern Pathology Core Facility using a standard H&E staining protocol.

Image Analysis

Imaging was conducted with DeltaVision RT Systems and SoftWorx software (Applied Precision, Inc., Issaquah, WA). For each section of tissue analyzed, at least 10 images were captured. Images were analyzed with SoftWorx and distances were measured using a two-point measuring tool included in the program. The superficial edge of the epithelium was visualized using either WGA or the background fluorescence of the tissue. To measure the keratin thickness, a perpendicular straight line was drawn from the surface to the basal edge of the layer defined by filaggrin expression (figure 1d). These two points were outlined using a combination of immunofluorescent markers (figure 1f). Sequential measurements were taken at even intervals along the tissue's surface. This was repeated every 30 µm along the epithelial surface to ensure adequate representation of the entire tissue's surface. We omitted all areas where the keratin was found separated from the epithelium. All measurements were taken in a blinded fashion (the party measuring the keratin thickness was unaware of the type of tissue being measured) and images were un-blinded after all measurements were recorded.

Statistical Analysis

A deviation approach analysis of variance (ANOVA) was utilized to compare mean keratin thickness of inner and outer foreskins. The full analysis included all of the measurements. Mean keratin thickness was computed for each subject per tissue. Given the variability of sample numbers in each subject a second analysis was computed using 20 randomly selected samples from each tissue per subject ("20-representative"). The computed means were the score for each of the 16 subjects per group. The distribution of means was analyzed for normality by visual inspection of normal Q-Q plots and the Shapiro-Wilk test. Heteroscedasticity was assessed roughly by comparing the ratio of variances and the Brown-Forsythe test. Assumptions of normality and homogeneity of variance were met to proceed with ANOVA. STATA ver. 10.1 (College Station, TX) was used for statistical analysis. A p-value of < 0.05 was considered statistically significant.

Results

Sixteen adult foreskins were obtained from consenting male donors. We did not collect clinical information from the subjects, but the most common medical indication for elective male circumcision was phimosis. We used fluorescent WGA to initially visualize the basic tissue structure (figure 1a). We then tested antibodies against a variety of proteins with known expression in the epithelium (including cytokeratins 8, 10, 13, and involucrin) and chose a filaggrin antibody to highlight the keratin layer (figure 1b) [7]. Filaggrin binds to keratin and helps keratin filaments aggregate in the superficial-most layers of the epidermis [8, 9]. This marker allowed us to visualize the stratum corneum of the epithelium, the equivalent of what has previously been defined as "keratin" [5, 6, 10]. This molecular definition of keratin based on filaggrin expression facilitated accurate measurement of the thickness of this layer and provided an alternative technique to evaluate foreskin keratin. The superficial strata were more clearly defined when the tissue was stained with both WGA and α -filaggrin, as shown in figure 1c. Grossly, there were no appreciable differences noted in the appearance of the inner as compared to the outer aspects of the foreskin.

In order to test our method of visualizing the keratin layer, we compared sections of foreskin taken from the same donor using the standard method of fixation and staining (Paraffin/H&E) to the OCT and immunofluorescence (OCT/Filaggrin) protocol outlined above. We additionally took sequential (side by side) sections from blocks of tissue preserved in either paraffin or OCT, and compared H&E to filaggrin staining for each type of medium (data not shown). Overall, we found that our methods allowed for enhanced visualization of the keratin layer. Figure 2 shows an example of inner foreskin using Paraffin/H&E stains (top) and OCT/Filaggrin stains (bottom). The keratin layer is more easily distinguished from the granular layer in the bottom image, using our fixing and staining methods. To quantify the differences between these two techniques, images of inner and outer foreskin were blinded and then analyzed as described in the methods section. The average thickness of the outer foreskin as evaluated by filaggrin expression was $15.8 \pm 8.18 \mu\text{m}$ while H&E staining gave a thickness of $14.8 \pm 5.58 \mu\text{m}$ ($p=0.995$). Therefore, both methods gave very similar values, demonstrating that any difference between our method and those of previous reports was not due to differences in tissue preparation, staining, or evaluation.

Neither method of tissue fixation was able to completely prevent against separation of the keratin layer in certain areas, a phenomenon that has been noted by other groups studying the structural characteristics of the male foreskin [6]. We excluded these desquamating areas in our analysis because keratin thicknesses could not be properly assessed. We did not find a significant difference between the inner and outer foreskin with regards to desquamation (data not shown).

Keratin thicknesses were measured as described in the methods section for the inner and outer aspects of each of the 16 specimens. In total, 2,032 measurements were obtained from the 16 subjects with a roughly balanced distribution (average of 62.1 and 64.9 measurements per inner and outer foreskin specimen, respectively). Repeated measurements were taken to account for variations in keratin layer thicknesses within a single specimen. However, there was significant variation in the relative contribution of samples. A secondary analysis was undertaken due to this variation, and is explained in the methods section and below.

There was no clear trend in the keratin thicknesses of the inner or outer foreskin. We observed significant heterogeneity between specimens obtained from different donors, as illustrated in figure 3. There was also notable intraindividual variability in the measured keratin thicknesses of some donors. For example, donor 8 had an interquartile range of 22.1 and 42.3 μm for the inner and outer foreskins, respectively. We therefore calculated p -values for each donor using the t-test that correlated with the heteroscedasticity of the data. We found that the keratin of the inner foreskin was thicker than that of the outer foreskin of seven donors; the outer foreskin was more thickly keratinized than the inner foreskin of three donors; and no significant difference was seen between the two aspects of six donor foreskins (table 1).

Boxplots of the inner and outer keratin thickness combining all donors showed substantial overlap between the keratinization of the two foreskin aspects (figure 4). The mean $\pm$ standard deviations of the keratin thickness of the inner and outer foreskin were 25.37 ± 12.51 and $20.54 \pm 12.51 \mu\text{m}$, respectively. This represented a very small difference of $\sim 5 \mu\text{m}$ between the inner and outer foreskin. The stratum corneum alone in a healthy individual can be up to 40 μm ; our measured difference is therefore only a small fraction of this layer, and much less the entire epidermal layer [11]. Analysis of variance with both the complete and 20-representative model showed a non-significant difference in mean keratin thickness between inner and outer foreskins ($p=0.451$ and 0.169 , respectively).

Discussion

Male circumcision has been shown to prevent HIV and other sexually transmitted infections (STIs) [12–17]. The dominant theories regarding the underlying mechanism of this protective effect focus on the keratin layer of the inner foreskin. During sexual intercourse, this thinner keratin layer potentially increases the exposure of target cells to HIV. However, studies concerning foreskin keratinization have been inconclusive and contradictory. Our study addresses this critical impasse by quantifying the keratin thickness of 16 foreskin specimens using a novel method for keratin measurements. We found no difference between the inner and outer foreskin keratin layers, invalidating the hypothesis that the thinner keratin barrier of the inner foreskin leads to increased HIV infection in uncircumcised men. Our findings are in agreement with Qin et al., who measured the keratin layer of foreskins from Chinese men and boys [6].

Previous studies have lacked description of the heterogeneous nature of the foreskin's keratin layer. We observed significant heterogeneity between the donors in our study, as seen in the measurements presented in figure 4 and table 1 which were determined with multiple samplings (>50/specimen). This phenomenon has been seen in dermatologic studies when skin samples are compared across a group of individuals, and may be related to genetic differences in skin composition [11,18–20]. In addition, exposure to environmental stimuli over an individual's lifespan might also induce changes in the expression of epidermal proteins, such as filaggrin or keratin.

In order to address potential differences in keratin thickness that may have been introduced by our methods, we conducted a detailed analysis of the methods used for fixing and staining the foreskin tissue. Previous analyses of foreskin keratinization have relied on formalin-fixed, paraffin-embedded tissue stained with H&E [2,5,6]. These stains are non-specific; they depend on differences in chemical composition, which may render it difficult to distinguish each epithelial stratum. Standard immunohistochemical stains against epidermal cytokeratins (including AE1/AE3 and CK5/6) are also non-specific for our area of interest—they stain for keratin production in basal layers of the epidermis [21]. The method developed by our group allows for high-resolution images based on the expression of proteins that highlight the keratin layer. In comparing sections from the same donor using different fixes and stains, we found no difference between the different methods, confirming the accuracy of our technique in measuring keratin thicknesses.

Although our study is the second largest to compare inner and outer foreskin keratinization, it remains limited by its small sample size. Future studies with a larger number of specimens (including penile specimens) will more clearly highlight similarities and differences between tissue types. Another drawback was the statistical analysis undertaken for this study, which may not completely take into account the intra-individual variability in measurements. Additionally, these measurements were taken from explanted tissue that is devitalized, though any effects to the keratin layer from this are likely minimal. Lastly, we did not know the donors' exact medical indication for male circumcision. Most donors undergo male circumcision for phimosis (difficulty retracting the foreskin), which is commonly due to recurrent episodes of balanitis (or inflammation of the foreskin due to infections or diabetes mellitus) [22]. It is unknown how these underlying conditions affect foreskin keratinization, and hence our findings. However, Qin et al. also evaluated the foreskins of healthy pre-school boys and found similar results [9]. We have also directly compared the foreskin to the corresponding penile tissue from two uncircumcised cadaveric donors and found only marginal differences in the keratin thickness between foreskin and penile epithelia (data not shown). Additionally, circumcised penile epithelia tended to be more thinly keratinized than uncircumcised penile

epithelia, further refuting the hypothesis that thinner keratin layers correspond to easier HIV transmission.

We propose that the mechanism responsible for the protective effect of male circumcision on HIV transmission involves more than keratin alone. A recent study by Kigozi et al. evaluating Ugandan men who underwent male circumcision described differences in HIV susceptibility that correlated with the size of their excised foreskin [23]. These findings suggest that the larger area of squamous epithelium provided by the presence of the foreskin may play a more important role in HIV transmission. Features such as epithelial permeability and inflammatory responses also vary widely and may have epidemiological associations with HIV infection [19,20,24]. Other studies have demonstrated a correlation between stricter preputial (the area between the foreskin and the glans penis) hygiene and lower HIV prevalence rates, suggesting that the dynamic environment created by the foreskin also plays an important role [25,26]. In addition, the results of the Merck HIV-1 gag/nef/pol vaccine trial imply that being uncircumcised alone did not confer the highest risk of HIV infection; vaccination further enhanced that risk in a way that was not evident in the serologic response to the vaccine [27]. These observations together argue against an overly simplistic model relating to keratin layers in the foreskin alone.

Ultimately, we need to better understand how HIV dynamically interacts with human genital mucosal surfaces, particularly in response to conditions that alter the mucosal environment created by the foreskin. Further studies aimed at clarifying these interactions will also help determine whether the protective effect of male circumcision, as seen in the African trials, can be applied to different at-risk groups, such as men who have sex with men. Only with a thorough understanding of the mechanism underlying the protective effects of male circumcision will it be possible to create effective topical agents or systemic vaccines to help finally stem the global epidemic.

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References

1. Donoval BA, Landay AL, Moses S, Agot K, Ndinya-Achola JO, Nyagaya EA, et al. HIV-1 Target Cells in Foreskins of African Men With Varying Histories of Sexually Transmitted Infections. *Anatomic Pathology* 2006;125:386–391.
2. McCoombe SG, Short RV. Potential HIV-1 target cells in the human penis. *AIDS* 2006;20:1491–1495. [PubMed: 16847403]
3. Hussain LS, Lehner T. Comparative investigation of Langerhans' cells and potential receptors for HIV in oral, genitourinary and rectal epithelia. *Immunology* 1995;85:475–484. [PubMed: 7558138]
4. Fischetti L, Barry SM, Hope TJ, Shattock TJ. HIV-1 infection of human penile explant tissue and protection by candidate microbicides. *AIDS*. 2008 In Press.
5. Patterson BK, Landay A, Siegel JN, et al. Susceptibility of Human Immunodeficiency Virus-1 Infection of Human Foreskin and Cervical Tissue Grown in Explant Culture. *American Journal of Pathology* 2002;161:867–873.
6. Qin Q, Zheng X, Wang Y, Shen H, Sun FWD. Langerhans' cell density and degree of keratinization in foreskins of Chinese preschool boys and adults. *International Urology Nephrology*. 2009
7. McGrath JA, JU. The filaggrin story: novel insights into skin-barrier function and disease. *Trends in Molecular Medicine* 2008;14:20–27. [PubMed: 18068483]

8. Sandilands A, Sutherland C, Irvine AD, WHM. Filaggrin in the frontline: role in skin barrier function and disease. *J Cell Sci* 2009;122:1285–1294. [PubMed: 19386895]
9. Gerritsen MJ, Elbers ME, de Jong EM, PCvdK. Recruitment of cycling epidermal cells and expression of filaggrin, involucrin and tenascin in the margin of the active psoriatic plaque, in the uninvolved skin of psoriatic patients and in the normal healthy skin. *J Dermatol Sci* 1997;14:179–188. [PubMed: 9138475]
10. Pask AJ, McInnes KJ, Webb DR, RVS. Topical oestrogen keratinises the human foreskin and may help prevent HIV infection. *PLoS ONE* 2008;3:e2308. [PubMed: 18523637]
11. Holbrook KA, GFO. Regional differences in the thickness (cell layers) of the human stratum corneum: an ultrastructural analysis. *J Invest Dermatol* 1974;62:415–422. [PubMed: 4820685]
12. Bailey RC, Moses S, Parker CB, et al. Male circumcision for HIV prevention in young men in Kisumu, Kenya: a randomised controlled trial. *Lancet* 2007;369:643–656. [PubMed: 17321310]
13. Tobian AA, Serwadda D, Quinn TC, Kigozi G, Gravitt PE, Laeyendecker O, et al. Male circumcision for the prevention of HSV-2 and HPV infections and syphilis. *N Engl J Med* 2009;360:1298–1309. [PubMed: 19321868]
14. Quinn TC. Circumcision and HIV transmission. *Current Opinions in Infectious Diseases* 2007;20:33–38.
15. Gray RH, Kigozi G, Serwadda D, Makumbi F, Nalugoda F, Watya S, et al. The effects of male circumcision on female partners' genital tract symptoms and vaginal infections in a randomized trial in Rakai, Uganda. *Am J Obstet Gynecol* 2009;200:42.e41–47. [PubMed: 18976733]
16. Auvert B, Taljaard D, Lagarde E, et al. Randomized, Controlled Intervention Trial of Male Circumcision for Reduction of HIV Infection Risk: The ANRS 1265 Trial. *PLoS Medicine* 2005;2:e298. [PubMed: 16231970]
17. Gray RH, Kigozi G, Serwadda D, Makumbi F, et al. Male circumcision for HIV prevention in men in Rakai, Uganda: a randomised trial. *Lancet* 2007;369:657–666. [PubMed: 17321311]
18. Russell LMWS, Delgado-Charro MB. The determination of stratum corneum thickness: an alternative approach. *Eur J Pharm Biopharm* 2008;69:861–870. [PubMed: 18424094]
19. Taylor SC. Skin of color: Biology, structure, function, and implications for dermatologic disease. *Journal of the American Academy of Dermatology* 2002;46:S41–S62. [PubMed: 11807469]
20. Wesley NO, HIM. Racial (ethnic) differences in skin properties: the objective data. *American Journal of Clinical Dermatology* 2003;4:843–860. [PubMed: 14640777]
21. Moll RDM, Langbein L. The human keratins: biology and pathology. *Histochem Cell Biol* 2008;129:705–733. [PubMed: 18461349]
22. Jordan, GH.; SMS. Wein: Campbell-Walsh Urology. Philadelphia, PA: Saunders Elsevier; 2007.
23. Kigozi G, Wawer M, Ssettuba A, Kagaayi J, Nalugoda F, Watya S, Mangen FW, Kiwanuka N, Bacon MC, Lutalo T, Serwadda D, Gray RH. Foreskin surface area and HIV acquisition in Rakai, Uganda (size matters). *AIDS* 2009;23:2209–2213. [PubMed: 19770623]
24. UNAIDS. AIDS Epidemic Update: December 2007. UNAIDS; 2007. UNAIDS/07.27E/JC1322E
25. O'Farrell N, Morson L, Moodley P, et al. Association Between HIV and Subpreputial Penile Wetness in Uncircumcised Men in South Africa. *Journal of Acquired Immune Deficiency Syndrome* 2006;43:69–77.
26. O'Farrell N, Morison L, Chung CK. Low prevalence of penile wetness among male sexually transmitted infection clinic attendees in London. *Sexually Transmitted Diseases* 2007;34:408–409. [PubMed: 17016235]
27. Buchbinder SP, Mehrotra DV, Duerr A, Fitzgerald DW, Mogg R, Li D, et al. Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial. *Lancet* 2008;372:1881–1893. [PubMed: 19012954]

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Table 1

Summary of Individual Donor Keratin Thicknesses

Summary of Measured Keratin Thicknesses of Individual Donor Specimens. Number of measurements taken per donor specimen, mean $\pm$ standard deviations, median and interquartile ranges of measured keratin thicknesses for inner and outer foreskin, listed by donor (corresponding to donor numbers in boxplots in figure 3). $p=0.451$ for mean thicknesses of inner and outer foreskin.

Donor #	Inner Foreskin					Outer Foreskin				
	Measurements Taken	Mean $\pm$ SD	Median	IQR	Measurements Taken	Mean $\pm$ SD	Median	IQR	Measurements Taken	Mean $\pm$ SD
1	85	20.5 $\pm$ 7.09	20.1	7.43	61	19.8 $\pm$ 5.61	18.9	7.45		
2	26	38.3 $\pm$ 11.0	36.1	16.5	51	19.5 $\pm$ 6.47	20.4	7.93		
3	88	33.2 $\pm$ 11.6	30.7	17.1	72	22.7 $\pm$ 8.61	22.3	12.2		
4	53	20.8 $\pm$ 5.87	19.4	9.68	75	23.3 $\pm$ 12.7	20	13		
5	29	44.2 $\pm$ 11.0	43.1	16.8	38	24.3 $\pm$ 4.15	23.7	4.9		
6	86	40.1 $\pm$ 10.9	39.8	16	84	21.1 $\pm$ 8.46	20	6.59		
7	51	20.9 $\pm$ 5.78	19.9	5.25	65	14.0 $\pm$ 8.05	11.8	6.34		
8	20	20.0 $\pm$ 11.4	21.3	22.1	20	33.0 $\pm$ 21.6	34	42.3		
9	77	30.7 $\pm$ 8.31	29.1	8.43	68	32.0 $\pm$ 18.2	32.9	32.6		
10	114	15.8 $\pm$ 5.81	15.6	8.52	110	15.6 $\pm$ 4.13	15.2	4.84		
11	33	47.4 $\pm$ 8.51	46.4	13.7	34	10.6 $\pm$ 3.48	10.6	4.56		
12	69	23.9 $\pm$ 7.70	21.3	7.48	153	14.9 $\pm$ 4.19	13.9	4.91		
13	31	24.3 $\pm$ 7.67	24.3	12.2	27	35.6 $\pm$ 14.0	34.4	29		
14	94	9.15 $\pm$ 3.87	8.72	6.12	48	9.65 $\pm$ 3.89	9.02	4.53		
15	38	8.28 $\pm$ 2.87	7.64	3.98	27	12.6 $\pm$ 5.95	11.3	5.54		
16	100	8.34 $\pm$ 1.80	8.24	2.09	105	20.0 $\pm$ 8.32	19.2	8.32		
Total	994	Mean=25.37			1038	Mean=20.54				

SD = standard deviation

IQR = interquartile range