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A Rapid Multiplex ARMS-PCR Method for the Detection of Four Single Nucleotide Polymorphisms of the Vitamin D Receptor (VDR) Gene

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Abstract: *The most studied single nucleotide polymorphisms of the VDR gene are Bsm1, Apa1, Taq1 and Fok1. Previously, many approaches have been used to study SNPs in VDR gene including restriction fragment length polymorphism (RFLP), Single Amplification refractory mutation system PCR (Single-ARMS-PCR), and sequencing of the VDR gene. The objective of the study was to develop a multiplex ARMS-PCR system for genotyping the four SNPs of the Vitamin D Receptor gene in just two simultaneous reactions. DNA samples of 353 individuals were genotyped for Bsm1, Apa1, Taq1 and Fok1 of the VDR gene SNPs using the Multiplex-ARMS-PCR system. Thirty two DNA samples were randomly selected and genotyped for the same polymorphisms with the Single-ARMS-PCR method, while genotyping with the Multiplex-ARMS-PCR was repeated for 37 DNA samples to verify for reproducibility. The Multiplex-ARMS-PCR system gave consistent results with the Single-ARMS-PCR system. The genotype frequencies of the Fok1 ploymorphism were FF (67.1%), Ff (28.9%) and ff (4.0%); for Bsm1 were BB (9.4%), Bb (38.8%) and bb (51.8%); Taq1 were TT (46.7%), Tt (47.3%) and tt (6.0%) and for Apa1 were AA (42.8%), Aa (40.5%) and aa (16.7%). The allelic frequency of 'F' versus 'f' was 82% versus 18%, 'B' versus 'b' was 29% versus 71%, 'T' versus 't' was 70% versus 30% and 'A' versus 'a' was 63% versus 37%. The Multiplex-ARMS-PCR technique gave consistent results throughout the study and was comparable when repeated separately using Single-ARMS-PCR method. This technique was found to be simple, reliable and less time consuming, thus could be a useful alternative to study VDR gene polymorphisms in routine clinical and resource-limited settings.*

1. Introduction

The discovery of Vitamin D gave a new insight into the usefulness and medical importance of sunlight. At the time of discovery, the key role of vitamin D was in calcium metabolism and homeostasis, thus playing a role in normal bone mineralization [1]. Since then, many more new non-classical functions of this vitamin have been discovered [2]. Amongst these functions include its control of the immune system, especially the T-cell signalling pathway [3] and regulation of cell growth. 1α,25-dihydroxyvitamin D3 ($1,25(OH)_2D_3$) inhibits T-cell proliferation in cell-mediated immune response, leading to its use as a therapeutic agent in some immune mediated diseases such as Type 1 diabetes mellitus (T1DM) and Crohn's disease [4,5]. Low levels of vitamin D have also been observed in infectious and non-infectious diseases of the lungs, emphasizing its role in disease modulation [6]. Lower levels of 25-hydroxyvitamin D3 ($25(OH)D_3$) have been consistently observed in tuberculosis patients when compared to controls [7] The role of vitamin D in the regulation of cell growth led to a lot of research on the relationship between vitamin D and various cancers, especially colorectal and prostate cancers [8,9].

The active metabolite of vitamin D is $1,25(OH)_2D_3$ which results from the enzymatic hydroxylation of $25(OH)D_3$ by the enzyme 1α-hydroxylase predominantly produced by the kidneys [10]. This metabolite is referred to as vitamin D hormone because its biological action is produced by binding to a nuclear receptor referred to as vitamin D receptor (VDR) of target cells like many hormones and acts as gene activator or repressor, thus controlling gene expression which leads to the

desired phenotype [11]. The VDR belongs to a nuclear receptor superfamily which includes the retinoid, thyroid and steroid hormones. Binding of $1,25(OH)_2D_3$ to VDR within the cell leads to a conformational change in the VDR structure, causing it to dimerize with the retinoid X receptor (RXR) forming a heterodimer [12]. This complex migrates to the nucleus of the cell and binds to a high affinity vitamin D response element (VDRE) located at the 5' upstream region of the target genes. Interaction of this VDRE bound complex with other co-activators and the basal transcription complex leads to the activation of the gene [13]. Thus the integrity of the VDR is very important for the biological activity of vitamin D.

The gene for the expression of the VDR is found on the long arm of chromosome 12. The gene is about 75kb long and has nine exons. The first exon contains the 5' untranslated region, while the ninth exon has the 3' untranslated region [14]. Over sixty polymorphisms of the VDR gene ranging from the promoter to the 3' untranslated region have been identified [15]. The highly polymorphic nature of the VDR gene has led to many studies to investigate some of these polymorphisms with many phenotypic characteristics, including disease and non disease states. The earliest and most common studied polymorphisms are the single nucleotide polymorphisms (SNPs) and have been related to the presence or absence of established endonuclease restriction sites as a result single nucleotide base changes. *Bsm*1, *Apa*1, *Taq*1 and *Fok*1 restriction site polymorphisms and their relationship to both infectious and non infectious disease states have so far been the most studied [16].

The *Fok*1 restriction site is furthest upstream on exon 2 and its polymorphism leads to a C-T change and also a change of the translation start site. The C allele denoted as 'F' (absence of restriction site) leads to a change of the translation initiation to the second AUG, leading to a protein that is shorter (424 instead of 427 amino acids) than the T allele denoted as 'f'. The *Bsm*1restriction site is found on intron 8 and a polymorphism yields a T-C change, referred to as T and C alleles respectively. The C allele (denoted as 'b') indicates presence of the restriction site while the T allele (denoted as 'B') depicts the absence of the restriction site. The *Apa*1 restriction site is also found on intron 8 and its polymorphism leads to a T-G change, with the T allele denoted as 'A' and the G allele as 'a'. The *Taq*1 restriction site is very close to the Apa1 site, though it is on exon 9 and its polymorphism leads to a T-C transition and equally the T allele is denoted as 'T' while the C allele which indicates the presence of the restriction site is depicted as 't' [12].

The increase in knowledge about the association between the VDR and its gene polymorphisms with many diseases has led to the development of many methods of identifying these polymorphisms. The earliest and common method of genotyping VDR gene polymorphisms was the use of PCR-RFLP. Primers were used to amplify the desired region of the gene and the specific restriction enzymes used to cut at its site if present. Later gel electrophoresis was performed to evaluate the presence or absence of a restriction site to indicate the presence or absence of polymorphism. This method is very expensive, laborious, time consuming and less specific because of variability of the restriction enzyme activity. Amplification refractory mutation system PCR (ARMS-PCR), which is allele specific was first described by Newton *et al*. [17] and is able to detect single base substitutions. This has led to improved specificity because allele specific primers will amplify DNA only in the presence of the allele. In the single-ARMS-PCR each SNP is analysed by the use of three primers which include a common primer and two allele specific primers. Two simultaneous reactions are setup with each reaction tube containing the common primer and one of the allele specific primers. This method gives more reliable results when compared with RFLP-PCR [18] and thus has been developed by some researchers for use in VDR gene polymorphism [19].

Advancement in technology leads to development of new methodologies or continuous improvement of already existing methodologies to make them more specific, affordable and less time consuming. This led us to develop a multiplex-ARMS-PCR technique to detect simultaneously four most studied SNPs of the VDR gene in just two simultaneous reactions using the same set of primers used before for single-ARMS-PCR. The objective was to produce a protocol that is simple, less expensive and less time consuming, and that can be widely applicable in resource limited settings.

2. Material and Methods

2.1 Study population

A cross-sectional study was conducted in Fako Division, Southwest Region of Cameroon. A total 372 informed consenting participants were recruited by convenient sampling within the months of July and October 2015. They included 112 (30.1%) males and 260 (69.9%) females. The age of the participants ranged between 35 and 85 years with a mean of 53.7±11.9 years. The study population consisted of Bantus and Semi-Bantus who constitute the two main ethnic groups in Cameroon.

2.2 Ethical consideration

Ethical clearance was obtained from the Faculty of Health Sciences Institutional Review Board (FHS-IRB), and an administrative clearance was obtained from the Southwest Regional delegation of Public Health. Signed informed consent was obtained from

every participant who willingly accepted to take part in the study, after adequate sensitization. The study was executed in compliance with approved ethical guidelines as outlined in the Helsinki Declaration.

2.3 Sample collection and processing

Venous blood (4mL) was collected from each participant into Ethylenediaminetetraacetic acid (EDTA) tubes. The blood was centrifuged at 2500 RPM for 2 minutes and the plasma transferred into tubes for storage at -80^{o}C. The packed cells were stored at -20^{o}C to be used for DNA extraction.

2.4 DNA extraction

DNA was extracted using the salting out method [20], with some modifications. Packed cells (0.5 mL) were added to 1mL of red cell lysing buffer (RCLB) in a 1.5 mL micro centrifuge tube, mixed gently for 1 minute, and centrifuged for 1 minute at 13,000 RPM. The supernatant was discarded and the step repeated 4 times. The WBC pellet was washed once in sterile double distilled (dd) water for 15 minutes, and later centrifuged for 1 minute at 13,000 RPM and the supernatant discarded. The WBC pellet was then resuspended in 240 µL of sterile dd water, 80 µL of 5x proteinase K buffer, 20 µL of proteinase K (20 mg/mL) and 60 µL of 10% SDS, mixed gently and incubated at 55 ^{o}C for 90 minutes and until there was complete digestion of proteins. After incubation the sample was spun for 5 seconds at 13,000 RPM and after it attained room temperature, 100 µL of 6M NaCl was added, votexed for 15 seconds and centrifuged at 13,000 RPM for 5 minutes. The supernatant was transferred into a new 1.5 mL eppendorf tube and the centrifugation process was repeated. The supernatant was again transferred into another 1.5 mL eppendorf tube and the DNA precipitated with 1mL of cold absolute ethanol, centrifuged for 1 minute at 13,000 RPM and supernatant discarded. Excess ethanol was blotted on a tissue and the step repeated twice instead with 70% ethanol. Finally, the pellets were dried and dissolved in 100 µL tris EDTA (TE) buffer for 15 minutes at 55 ^{o}C. The concentration and purity of the DNA was evaluated by a Nanophotometer (IMPLEN GmbH, Germany).

2.5 Multiplex-ARMS-PCR analysis

The procedure involved evaluation of all the four different polymorphisms (*Bsm*1 B/b, *Apa*1 A/a, *Taq*1 T/t and *Fok*1 F/f) in just two reaction tubes, one tube to detect all the wild-type (denoted by the capital letters B, A, T and F) and in the other tube all the mutant (b, a. t and f). The primers used were those predesigned by Jafari *et al*.[19] for the single-ARMS-PCR (Table 1). The primers were (produced by MOLEQULE-ON, New Zealand) reconstituted into a working stock of 10 mM. There were three primers for each polymorphism consisting of a common primer and two allele specific primers, one for each allele.

Gene amplification was done by using GoTaq Green Master Mix (Promega Inc., USA) containing Taq DNA polymerase, reaction buffer of pH 8.5, 400 µM dNTPs each, 3 mM $MgCl_2$ and nuclease-free water. The reaction was further optimized with additional 2 mM $MgCl_2$. The final optimization procedure to make up a total reaction volume of 25µL in each tube contained all the reagents as given in Table 2

The entire mixing of reagent took place on ice to prevent formation of primer dimmers.

Table 1: Primer sequences used in the Multiplex ARMS-PCR assay

Primer	Annealing temperature (^{o}C)	Amplicon size (bp)
FokI: rs10735810*		
FokI/F 5'TGGCCGCCATTGCCTCCG 3'		
FokI/f	62	
5'TGGCCGCCATTGCCTCCA 3'		77
FokI/C 5'AGCTGGCCCTGGCACTGA 3'	60	
	60	
BsmI: rs1544410*		
BsmI/B 5'AGCCTGAGTACTGGGAATGT 3'	60	
BsmI/b 5'AGCCTGAGTACTGGGAATGC 3'	62	534
BsmI/C 5'GGGAGGGAGTTAGGCACC 3'	60	
TaqI: rs731236*		
TaqI/T 5'CAGGACGCCGCGCTGATT 3'		
TaqI/t	62	
5'CAGGACGCCGCGCTGATC 3'		148
TaqI/C 5'CCTCATTGAGGCTGCGCAG 3'	60	
	62	
ApaI: rs7975232*		
ApaI/A 5'TGGGATTGAGCAGTGAGGT 3'	58	
ApaI/a 5'TGGGATTGAGCAGTGAGGG 3'	60	229
TaqI/C 5'CCTCATTGAGGCTGCGCAG 3'	62	

* SNP identification number as retrieved from dbSNP

C: indicates a common primer, the capital and lowercase letters represent the allele-specific primers.

To avoid errors in pipetting, larger volumes of the primers and $MgCl_2$ were prepared and aliquoted into the individual PCR tubes before adding the template DNA and lastly, master mix was added just before the tubes were placed in the thermocycler.

The target sequences were amplified in a thermocycler with a hot lid (GE9612-S Thermocycler BIO-GENER Technology, China). The amplification conditions were as follows: Initial denaturation step for 2 minutes at 95^{o}C, 29 cycles of

denaturation at 95°C for 25 seconds, annealing at 58°C for 30 seconds and extension at 72°C for 1minute; and a final extension at 72°C for 5 minutes. 10µL of the PCR product was subjected to electrophoresis on a 1.5% agarose gel impregnated with 5% Ethidium bromide in a 1x Tris borate EDTA (TBE) buffer at 80 volts for 40 minutes using Sub-cell GT agarose gel electrophoresis system (BIO-RAD, USA). Tubes containing the wild-type and mutant primers were placed on the gel next to each other. A 100 bp DNA ladder was included with each gel to serve as marker for DNA size. The gel was visualised in a UV gel doc (Gel Doc ™ 2000 BIO-RAD, USA).

Table 2: Reagents and primers mix specificities per reaction tube for the Multiplex ARMS-PCR assay

Reagent	Volume (concentration)
Master Mix	12.5µL
$MgCl_2$	1µL (2mM)
Bsm1 primers (Bsm1/C + B/b)	0.6µL (0.24µM) of each
Apa1 primers (Apa1/C + A/a)	0.25µL (0.1µM) of each
Taq1 primers (Taq1/C + T/t)	0.17µL (0.07µM) of each
Fok1 primers (Fok1/C + F/f)	0.17µL (0.07µM) of each
Template DNA	~10ng/µl
Nuclease free water	Variable, depending on volume of template DNA used.

Authentication of the results was done by performing single-ARMS-PCR for the individual polymorphisms of 32 randomly selected DNA samples using the same optimized conditions as the multiplex-ARMS-PCR and comparing the results with that obtained by the multiplex-ARMS-PCR. Also multiplex-ARMS-PCR was repeated for 37 randomly selected samples to assess for consistency and reproducibility of the method

2.6 Statistical analysis

The data was entered into EXCEL, and analysed with SPSS for windows version 20 (SPSS Inc., USA). Frequencies of the different polymorphisms were expressed in numbers and percentages. Chi square test was used to evaluate consistency of genotype distributions with Hardy-Weinberg equilibrium as well as between the two ethnic groups and previous studies with similar data. Statistical significance was set at $P \leq 0.05$.

3. Results

The Multiplex-ARMS-PCR was successfully performed in 353 DNA samples, 19 samples were disqualified because of either very low yield or poor quality of the DNA. There was 100% consistency of the results obtained between the multiplex-ARM-PCR and the single-ARMS-PCR and also following repeated rounds of the multiplex-ARM-PCR gave the same results, indicating that the methodology was specific and reproducible. Sample of the multiplex-ARM-PCR on agarose gel impregnated with Ethidium bromide and viewed under UV is presented in Figure 1.

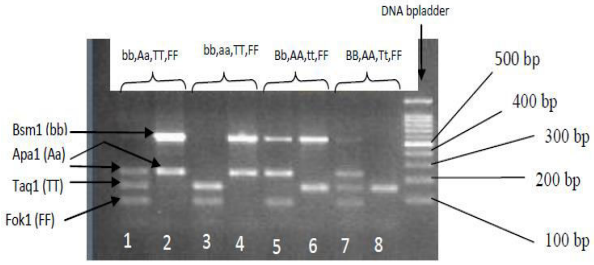

Figure 1: Multiplex ARMS-PCR electrophoresis gel image of results of some samples visualized in a UV Gel Doc. Lane 1 and 2, 3 and 4, 5 and 6, 7 and 8 consist of same samples. In the even number lanes, primers of mutant alleles and the in odd number lane, primers of wild type alleles were added. A band near 500 bp in only lane 2 represents *Bsm*1 SNP with bb genotype, two bands between 200 and 300 bp in lane 1 and 2 represents *Apa*I SNP with Aa genotype, one band between 100 and 200 bp in lane 1 represents *Taq*I SNP with TT genotype while last band below 100 bp in lane 1 represents presence of *Fok*I SNP with FF genotype. The collective genotypes of all SNP in lane 1 and 2 are as bb, Aa, TT and FF. The collective genotypes of remaining lanes are also mentioned in the top of this figure.

The frequencies of polymorphisms and the allelic frequencies agreed with the Hardy–Weinberg equilibrium (Table 3). There was no significant difference observed between gender and ethnic origin with all different polymorphisms in this study (Table 4).

The genotype proportions were compared to those obtained from previous studies within and out of Africa. There was no significant difference of the *Fok*1 genotype distribution between our study and the black African populations, while there were significant differences when compared with the white populations (Table 5). Also there was no significant difference of the *Fok*1 genotype between our study and African American population, indicating their black African ancestral origin. Only *Fok*1 genotype frequency showed a consistent association with the black African origin. There was a significant difference of the Fok1 genotype with the Egyptian population (P = 0.007), though African but not of the black race.

Table 3: *Fok*1, *Bsm*1, *Apa*1, and *Taq*1 Genotype and Allele frequency distributions of the Study population

Polymor-phism	N	Genotype Frequency N (%)	
*Fok*1	**353**	**FF**	**Ff**
		237 (67.1)	**102 (28.9)**
*Bsm*1	**353**	**BB**	**Bb**
		33 (9.4)	**137 (38.8)**
*Taq*1	**353**	**TT**	**Tt**
		165 (46.7)	**167 (47.3)**
*Apa*1	**353**	**AA**	**Aa**
		151 (42.8)	**143 (40.5)**

There was no significant difference in the *Bsm*1 genotype distribution between our study and the other studies, irrespective of population. For the *Taq*1 polymorphism, only the Egyptian population showed a significant difference (P = 0.012) in the genotype distribution with our study. In addition, only the South African (P < 0.001) and the English (P = 0.009) populations showed a statistically significant difference in the genotype distribution of *Apa*1 polymorphism with our study.

Table 4: Genotype distribution according to the two main ethnic groups of the study population

SNP genotype	Bantu N (%)	Semi-Bantu N (%)	χ^2 test	P-value
*Fok*1				
FF	84 (65.1)	149 (68.7)	2.775	0.25
Ff	42 (32.6)	57 (26.3)		
Ff	3 (2.3)	11 (5.1)		
*Bsm*1				
BB	16 (12.4)	17 (7.8)	1.965	0.37
Bb	48 (37.2)	86 (39.6)		
Bb	65 (50.4)	114 (52.5)		
*Apa*1				
AA	58 (45.0)	90 (41.5)	3.159	0.17
Aa	56 (43.4)	85 (39.2)		
aa	18 (11.6)	42 (19.4)		
*Taq*1				
TT	55 (42.6)	107 (49.3)	1.447	0.49
Tt	66 (51.2)	88 (45.2)		
tt	8 (6.2)	12 (5.5)		

4. Discussion

The aim of this study was to design a multiplex-ARMS-PCR system for the four most studied VDR SNPs (*Fok*1, *Bsm*1, *Apa*1, and *Taq*1) that was less time consuming, relatively reduced cost, easy to perform and that could be comparable with other established methods. The multiplex-ARMS-PCR method gave reliable and reproducible results when compared to the single-ARMS-PCR method used for VDR genotyping. The same primer sets were used in this study that were previously used for single-ARMS-PCR by Jafari *et al*.[19]; and they also showed that the single-ARMS-PCR was more reliable than RFLP-PCR for genotyping these SNPs. Since all the different polymorphisms could be evaluated in just two simultaneous reactions, it reduced the cost and the time required to obtain the results by four. A premix of the primers could actually be made and frozen to still reduce the manipulation time and avoid pipetting errors. Also many more samples (up to 46) could be analysed per batch if the thermocycler can hold up to 96 PCR tubes. Results could be interpreted by simple visualization of the gel under UV with a 100 bp DNA ladder as a DNA size marker, thus requiring less expertise. This method is very simple, economical and less time consuming as compared to single-ARMS-PCR, RFLP and sequencing protocols and thus can be performed under less sophisticated conditions as obtains in many African countries.

With this methodology, molecular diagnosis of VDR gene SNPs related to diseases can be performed in routine clinical diagnostic laboratories, even in resource limited settings and in the field after some adaptations.

There was no difference in the genotype distribution between the two ethnic groups involved in the study indicating little genetic variation within the population with respect to the VDR gene. We compared the genotype distribution of our results (*Bsm*1, *Apa*1, *Taq*1 and *Fok*1) with other studies, including the Iranian study that designed the primers that we used (Jafari *et al*., 2013) [19]. It was revealed that the *Fok*1 genotype was similar with all studies that involved the black African race and thus could be used to trace black ancestral lineage. This similarities of the *Fok*1 genotype frequency has previously been reported by Zmuda *et al*. in 2000[28]. Also the difference observed in the *Fok*1 genotype frequency of our study with other non-black populations is similar to results observed between blacks and whites in South Africa (O Neill *et al*., 2013) [29]. This finding could be exploited to create a better understanding of the link of the *Fok*1 genotype with certain disease susceptibility that is black race dependent. This information may be important since the *Fok*1 polymorphism actually leads to a change in the size of the VDR.

5. Conclusion

The result of the genotype obtained by the multiplex-ARMS-PCR was the same when compared to that of the single-ARMS-PCR. This method is reliable,

simple to perform, less time consuming and also less costly. The method could therefore be exploited and adapted in resource-limited settings like most African countries and also where less technical expertise is available. It can also be used for routine genotyping in clinical settings.

6. Acknowledgements

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7. Ethical approval

"All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards."

Table 5: Comparison of VDR gene polymorphisms distribution between our study population and other published data

Study	**Population**	**Genotype (%)**				**P-value**
		N	**FF**	**Ff**	**ff**	
Our study	Cameroonian	353	67.1	28.9	4.0	Ref
Bornman *et al.*[21]	West African*	1134	61.9	33.5	4.6	0.74
Soborg *et al.*[22]	Tanzanian	426	64	31	5	0.89
Lombard *et al.*[23]	South African	117	76.7	21	2.3	0.31
Rizk *et al.*[24]	Egyptian	212	46.2	42.5	11.3	0.007
Sarkissyan *et al.*[25]	African American	150	61.3	32.7	6.0	0.64
Jafari *et al.*[19]	Iranian	218	43	52	5	0.003
Fernandez-Mestre *et al.*[26]	Venezuelan	195	30.7	54.9	14.4	< 0.001
		No	**BB**	**Bb**	**bb**	
Our study	Cameroonian	353	9.4	38.8	51.8	Ref
Bornman *et al.*[21]	West African*	977	6.1	32.3	61.6	0.34
Lombard *et al.*[23]	South African	117	8	27.3	64.7	0.17
Sarkissyan *et al.*[25]	African American	151	11.9	49.0	39.1	0.20
Jafari *et al.*[19]	Iranian	218	12	43	45	0.60
		No	TT	Tt	tt	
Our study	Cameroonian	353	46.7	47.3	6.0	Ref
Bornman *et al.*[21]	West African*	977	51.7	39.4	8.9	0.46
Søborg *et al.*[22]	Tanzanian	426	56	38	5	0.38
Lombard *et al.*[23]	South African	117	57.3	41.5	1.2	0.10
Rizk *et al.*[24]	Egyptian	210	29.5	54.8	15.7	0.012
Sarkissyan *et al.*[25]	African American	147	57.1	32	10.9	0.07
Jafari *et al.*[19]	Iranian	218	46	48	6	0.99
Fernandez-Mestre *et al.*[26]	Venezuelan	183	59.6	38.8	1.6	0.08
		No	AA	Aa	aa	
Our study	Cameroonian	353	42.8	40.5	16.7	Ref
Bornman *et al.*[21]	West African*	977	42.8	45.5	11.7	0.56

Soborg *et al*.[22]	Tanzanian	426	50	40	10	0.33
Lombard *et al*.[23]	South African	117	24.3	72	3.7	< 0.001
Rizk *et al*.[24]	Egyptian	210	31.9	56.2	11.9	0.08
Sarkissyan *et al*.[25]	African American	147	49.0	41.5	9.5	0.30
Jafari *et al*.[19]	Iranian	218	30	45	25	0.13
Fernandez-Mestre *et al*.[26]	Venezuelan	190	29.5	50.5	20	0.15
Mulligham *et al*.[27]	English	101	23	50	27	0.009

*West African study included Gambia, Guinea and Guinea-Bissau

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