Comparative molecular genetics of odontogenic keratocysts in sporadic and syndromic patients

Melvin A. Ambele[1,2*], Liam Robinson[1], Marlene B. van Heerden[1], Michael S. Pepper[2], Willie F. P. van Heerden[1]

[1]Department of Oral Pathology and Oral Biology, School of Dentistry, Faculty of Health Sciences, University of Pretoria, Pretoria, South Africa

[2]Institute for Cellular and Molecular Medicine, Department of Immunology, and SAMRC Extramural Unit for Stem Cell Research and Therapy, Faculty of Health Sciences, University of Pretoria, Pretoria, South Africa

*Corresponding author:
Melvin A. Ambele
Department of Oral Pathology and Oral Biology, School of Dentistry, Faculty of Health Sciences, University of Pretoria, PO Box 1266, Pretoria, South Africa
Tel.: +27 (0) 73 648 0419
melvin.ambele@up.ac.za

Abstract

Odontogenic keratocysts (OKCs) are common cysts of odontogenic origin which usually occur as a single non-syndromic cyst in isolation (sporadic) or as syndromic multiple cysts as a manifestation of naevoid basal cell carcinoma syndrome (NBCCS). Alterations involving the *PTCH* gene are the most common identified factor associated with up to 85% and 84% of NBCCS and sporadic cases, respectively. Other Hedgehog (HH) pathway and non-HH pathway-associated genes have also been implicated in the pathogenesis of OKCs. This pilot study used the Affymetrix OncoScan® molecular assay to perform a comparative genomic analysis between four sporadic and three syndromic cases of OKC to identify molecular drivers that may be common and/or distinct in these two groups. The majority of alterations detected in both groups were CNN-LOH. Despite distinct molecular signatures shown by both groups, CNN-LOH alterations involving chromosome 9q affecting not only *PTCH* but also *NOTCH1* genes were detected in all syndromic and three sporadic cases. LOH alterations involving 16p11.2 affecting genes not previously described in OKCs were also detected in all syndromic

and 3 sporadic cases. Furthermore, alterations on 22q11.23 and 10q22.1 were also detected in both groups. Of note, alterations on 1p13.3, 2q22.1, and 6p21.33 detected in sporadic cases were absent in all syndromic cases. This study demonstrates that a more common group of genes may be affected in both groups of OKCs, whereas other alterations may be useful in distinguishing sporadic from syndromic cysts. These findings should be validated in larger OKC cohorts to improve molecular diagnosis and subsequent patient management.

Keywords: Affymetrix OncoScan molecular assay; odontogenic keratocysts; Gorlin–Goltz syndrome; molecular genetics; naevoid basal cell carcinoma syndrome; oral cancer

Introduction

Odontogenic keratocyst (OKC) is defined by the 2022 WHO Classification as a developmental odontogenic cyst that is characterised histologically by a thin parakeratinised stratified squamous epithelial lining with palisaded and hyperchromatic basal cells and clinically by a tendency to recur after treatment[1,2]. OKCs are common cystic lesions of the jawbones that usually occur in isolation as a single non-syndromic cyst. In rarer instances, they present as multiple cysts with features of naevoid basal cell carcinoma syndrome (NBCCS), also known as Gorlin–Goltz syndrome (GGS)[3]. NBCCS is a rare autosomal dominant syndrome with high penetrance and variable expression. Clinical manifestations of the syndrome include multiple basal cell carcinomas, OKCs of the jaws, palmar or plantar pits, calcification of the falx cerebri, and other multisystem abnormalities involving the eyes, skeletal, neural, and reproductive systems[3-5]. Subsequently, numerous clinical and molecular studies have shown that there is a wide range of possible clinical features with variability in clinical presentation[6]. About 65-100% of NBCCS patients present with multiple OKCs throughout life[3]. In the vast majority of cases, these are multiple and present in a synchronous and metachronous manner. The number of cysts that each patient may have over a lifetime range from 1 to 28, with an average of 4 to 6[6]. These syndromic cysts are found in equal frequencies in both jaws, in contrast to non-

syndromic cysts, which are most frequently seen in the mandible[7].

Mutations in the genetic locus 9q22.3-q31, involving the *PTCH* gene, in particular, have been strongly associated with NBCCS[8,9]. *PTCH1*, a negative regulator of the Hedgehog (HH) pathway, has been detected in approximately 40-85% of NBCCS patients[10-12]. Alterations in the *PTCH1* gene have also been demonstrated in sporadic OKCs, but most cases have only involved one copy of the gene resulting in haploinsufficiency and reduced expression of the PTCH protein at the cell surface, with activation of the HH pathway. Alterations of the *PTCH* gene may be by somatic mutation, LOH or due to gene silencing by DNA methylation[13-15]. *PTCH1* gene alterations have been identified in up to 85% of OKCs associated with NBCCS and 84% of sporadic cysts[11,12]. A more recent study by Stojanov et al. found *PTCH1* inactivating mutations in 93% of sporadic OKCs, with biallelic inactivation in 80% of cases and 9q copy neutral loss of heterozygosity targeting the *PTCH1* locus in 15% cases[12]. Other studies also showed upregulation of the HH pathway associated genes *SMO*, *PTCH1*, *SUFU*, and *GLI1* as well as overexpression of the downstream target genes for cyclin D1 and bcl-2[16-18]. Mutations in OKCs are not limited to genes involved in the HH pathway, with other mutations involving genes such as *CDKN2A*, *TP53*, *MCC*, *CADMI* and *FHIT* reported[12,19-21].

The Affymetrix OncoScan® assay is a robust molecular technique used for investigating whole genome copy number alterations (CNAs), loss of heterozygosity (LOH) and somatic mutations in well-known genes that are important in cancer and tumour progression[22]. This study aimed to use the Affymetrix OncoScan® assay to investigate and characterise genomic alterations in seven cases of OKC, four sporadic cases and three in patients with NBCCS, to identify other molecular changes that may be common in and/or distinct between these two groups. This may provide new information that could improve prognosis and clinical management of these conditions based on their molecular profile.

Materials and Methods

Ethical statement

The study was conducted following approval by the University of Pretoria, Faculty of Health Sciences Research Ethics Committee (Reference number: 44/2010). All procedures followed the ethical standards of the Helsinki Declaration of 1975, as revised in 2008. All eight participants included in this study gave written informed consent.

Case selection

For this study confirmed cases of sporadic and syndromic odontogenic keratocysts were collected from the histopathological archives of the Department of Oral Pathology and Oral Biology, University of Pretoria. An experienced Oral and Maxillofacial Pathologist (W.F.P.vH) confirmed the diagnosis of all included cases. A diagnosis of NBCCS required two major and one minor diagnostic criterion or one major and three minor diagnostic criteria for inclusion in the study[5].

DNA isolation and OncoScan® FFPE Assay

Genomic DNA (gDNA) was isolated from formalin-fixed paraffin-embedded (FFPE) specimens from each case using the QIAamp DNA FFPE Tissue kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's protocol. The Quant-iT^{TM} PicoGreen® dsDNA Assay Kit (Life Technologies, Johannesburg, South Africa) was used to quantify gDNA. Following this, 80ng of gDNA from each sample was used to perform the Affymetrix OncoScan® FFPE assay (Affymetrix; ThermoFisher Scientific company) as per the manufacturer's protocol[23]. Fluorescence intensity (CEL) files generated from each scanned OncoScan® array were imported and processed using the Affymetrix OncoScan^{TM} console software (version 1.3) to generate OncoScan CHP files. These files were used for analysis of genome wide copy number alterations (CNAs), loss of heterozygosity (LOH), and 74 clinically

actionable somatic mutations in 9 cancer genes (*BRAF*, *EGFR*, *IDH1*, *IDH2*, *KRAS*, *NRAS*, *PIK3CA*, *PTEN*, and *TP53*)[22].

Results

Case classification

Four sporadic cases and three syndromic cases were selected for this study. Table 1 summarises the main demographic and clinical features of the cases included.

Table 1. Included sporadic and syndromic cases of odontogenic keratocysts

Sporadic cases	**Case 1**	**Case 2**	**Case 3**	**Case 4**
Age	20	39	13	26
Sex	M	F	M	F
Site	Posterior mandible	Posterior mandible	Anterior mandible	Posterior mandible
Singular/multiple cysts	Single	Single	Single	Single
Syndromic cases	**Case 1**	**Case 2**	**Case 3**	
Age	10	13	17	
Sex	M	F	M	
Site	Anterior maxilla and mandible; posterior mandible	Posterior mandible	Anterior maxilla and mandible; posterior mandible	
Singular/multiple	Multiple	Multiple	Multiple	

Histopathological analysis of cases

All cases, regardless of whether non-syndromic or syndromic, showed features of an odontogenic cyst lined by thin, parakeratinised epithelium 5-8 cell layers thick with palisading hyperchromatic basal cells (Figure 1A). The fibrous connective tissue cyst wall showed varying degrees of inflammation amongst the cases. Some cases showed satellite cysts and solid islands in the wall and budding of the basal epithelial layer (Figure 1B). These features were more common and prominent in cysts associated with syndromic patients.

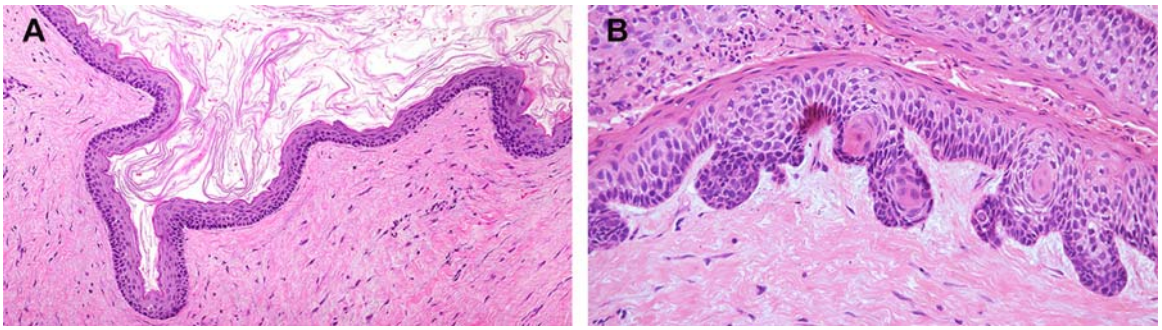

Figure 1. Hematoxylin & eosin micrograph of odontogenic keratocysts. (A) Characteristic lining of a nonsyndromic odontogenic keratocyst at original magnification ×200 and (B) Odontogenic keratocyst from a syndromic patient showing extensive budding of the basal epithelial layer at original magnification ×400.

Genetic alterations detected in sporadic OKCs

Table 2 summarises the main genetic alterations detected in the sporadic cases of OKC included in this study. About 68% (17 out of 25) of all the genetic alterations found in sporadic OKCs cases were CNN-LOH (Table 2). Three of four cases showed CNN-LOH on the entire chromosome 9q containing the *PTCH* gene. LOH on 16p11.2 was detected in cases 2, 3 and 4. On the other hand, CNN-LOH on 2q22.1 (*THSD7B, HNMT, SPOPL, NXPH2, YY1P2* and *LRP1B*) was detected in cases 1 and 2, and CNN-LOH on 6p21.33 (*DDX39B, ATP6V1G2-DDX39B, SNORD117, SNORD84, ATP6V1G2, NFKBIL1, LTA, TNF, LTB, LST1, NCR3, AIF1, PRRC2A, SNORA38, BAG6, APOM, C6orf47, GPANK1, CSNK2B, LY6G5B, LY6G5C, ABHD16A, MIR4646, LY6G6F, LY6G6E, LY6G6D, LY6G6C, C6orf25, DDAH2, CLIC1, MSH5, MSH5-SAPCD1, SAPCD1, VWA7, VARS, LSM2, HSPA1L, HSPA1A, HSPA1B, C6orf48, SNORD48, SNORD52, NEU1, SLC44A4, EHMT2, C2, ZBTB12, CFB, NELFE, MIR1236, SKIV2L, DOM3Z, STK19, C4A, C4B, C4B_2, CYP21A2, CYP21A1P, TNXA, TNXB, ATF6B, FKBPL, PRRT1, LOC100507547, PPT2, PPT2-EGFL8, EGFL8, AGPAT1, RNF5, RNF5P1, AGER, PBX2, GPSM3, NOTCH4, C6orf10, HCG23, BTNL2, HLA-DRA, HLA-DRB5, HLA-DRB6, HLA-DRB1, HLA-DQA1, HLA-DQB1, HLA-DQA2, HLA-DQB2, HLA-DOB, TAP2, PSMB8, LOC100507463, TAP1, PSMB9, LOC100294145, HLA-DMB, HLA-*

DMA, BRD2, HLA-DOA, HLA-DPA1, HLA-DPB1, HLA-DPB2, COL11A2, RXRB, SLC39A7, HSD17B8, MIR219-1, RING1, HCG25, VPS52, RPS18, B3GALT4, WDR46, PFDN6, RGL2) was detected in cases 2 and 4.

Table 2. Genetic alterations detected on chromosomal locations in sporadic cases

Genetic alterations	**Case 1**	**Case 2**	**Case 3**	**Case 4**
1p13.3; 37.093 **(2)**			Gain	
1p31.3; 2660.465 **(13)**				CNN-LOH
1q31.3; 348.132 **(7)**	Loss			
2q21.2; 24180.875 **(61)**		CNN-LOH		
2q22.1; 4715.125 **(6)**	CNN-LOH			
2q32.3; 4196,662 **(10)**				CNN-LOH
3p21.31; 4219.184 **(129)**				CNN-LOH
3q26.1; 253.637 **(0)**	Loss			
5p12; 2552.117 **(17)**		CNN-LOH		
6p21.33; 2899.352 **(137)**		CNN-LOH		
6p22.2; 6884.866 **(270)**				CNN-LOH
8q11.21; 2935.736 **(4)**		CNN-LOH		
Entire 9q**; (675) *PTCH***	CNN-LOH		CNN-LOH	CNN-LOH
10q22.1; 2548.51 **(38)**		CNN-LOH		
10p15.3; 439,259 **(1)**			Gain	
11p11.2; 3686.407 **(18)**				CNN-LOH
16p11.2; 3400.557 **(16)**		Loss+LOH		
16p11.2; 2067.992 **(8)**		Loss		
16p11.2; 3314.358 **(15)**			CNN-LOH	
16 p11.2; 2628.54 **(12)**				CNN-LOH
16q23.2; 2578,918 **(18)**			CNN-LOH	
22q11.23; 43.891 **(4)**	Loss			
22q11.23; 1211.192 **(35)**				Gain

**The bold text represents the chromosome cytoband start location, followed by the size (kbp) of the mutation, and the number of genes affected in parenthesis. LOH (loss of heterozygosity) and CNN (copy number neutral)*

Several regions showed genetic alterations in single cases, alluding to the uniqueness of each case. Such uniqueness per individual case includes loss of 1q31.3, 3q26.1 and 22q11.23 detected in case 1. Case 2 had CNN-LOH on 5p12, 8q11.21, and 10q22.1. Case 3 showed gain in 1p13.3 and 10p15.3, and CNN-LOH on 16q23.2. Case 4 showed CNN-LOH on 1p31.3, 2q32.3, 3p21.31, and 11p11.2, and a gain on 22q11.23. The affected genes in these unique genetic alterations are listed in supplementary Table S1.

Genetic alterations detected in syndromic OKCs

Table 3 summarises the main genetic alterations detected in the syndromic cases of OKC included in this study. Approximately 62% (16 out of 26) of genetic alterations detected in syndromic cases represented a CNN-LOH, with each case showing some degree of uniqueness in genetic alterations not detected in others (Table 3). A CNN-LOH on the entire chromosome 9q containing the well-described *PTCH* gene involved in NBCCS was detected in all three cases. Furthermore, LOH on 16p11.2 (*TP53TG3, TP53TG3B, TP53TG3C, SLC6A10P, LOC390705, RNU6-76P, LINC00273*) was also common to all three NBCCS cases. None of these affected genes have been previously described in this syndrome. Gains on 22q11.23 (*GSTTP1, LOC391322, GSTT1, GSTTP2*) were common to 2 of 3 cases.

The uniqueness of the molecular profile of each case includes loss at 3p26.2, 14q32.33, and 19p13.3, and CNN-LOH on 11p11.12 in case 1. Case 2 showed CNN-LOH on 2q21.3, 2q24.2, 3p21.31, 9p23, 10q11.21, and 12q21.31, a loss on 2q37.3, and a gain on 14q11.2. Case 3 showed CNN-LOH on 2p12, 10q22.1, 13q32.1, and 14q23.3, and a loss on 10q11.22. The affected genes in these unique genetic alterations are listed in supplementary Table S2.

Table 3. Genetic alterations detected on chromosomal locations in syndromic cases

Genetic alterations	Case 1	Case 2	Case 3
2p12; 6047.015 **(29)**			CNN-LOH
2q21.3; 2727.299 **(14)**		CNN-LOH	
2q24.2; 3429.454 **(14)**		CNN-LOH	
2q37.3; 272.47 **(3)**		Loss	
3p21.31; 4137.515 **(109)**		CNN-LOH	
3p26.2; 254.587 **(0)**	Loss		
9p23; 7193.589 **(28)**		CNN-LOH	
Entire 9q**; (675)** ***PTCH***	CNN-LOH	CNN-LOH	CNN-LOH
10q11.21; 2733.39 **(32)**		CNN-LOH	
10q11.22; 950.187 **(12)**			Loss
10q22.1; 2524.995 **(38)**			CNN-LOH
11p11.12; 2609.203 **(10)**	CNN-LOH		
12q21.31; 3550.732 **(10)**		CNN-LOH	
13q32.1; 2670.047 **(11)**			CNN-LOH
14q11.2; 352.713 **(9)**		Gain	
14q23.3; 2723.579 **(20)**			CNN-LOH
14q32.33; 252.461 **(1)**	Loss		
16p11.2; 2806.776 **(21)**	LOH		
16p11.2; 909.866 **(6)**	Gain		
16p11.2; 3705.697 **(19)**		CNN-LOH	
16p11.2; 2742.561 **(13)**			CNN-LOH
19p13.3; 57.364 **(7)**	Loss		
22q11.23; 50.171 **(4)**	Gain		
22q11.23; 43.891 **(4)**			Gain

**The bold text represents the chromosome cytoband start location, followed by the size (kbp) of the mutation, and the number of genes affected in parenthesis. LOH (loss of heterozygosity) and CNN (copy number neutral)*

Common genetic alterations in sporadic and syndromic OKCs

Three genetic alterations were found to be common between the two groups of OKCs. The first was a CNN-LOH involving the entire long arm of chromosome 9, which contains the *PTCH* gene. Other cancer-related genes such as *NOTCH1* also located on the affected region of chromosome 9 may contribute to the development of both types of OKCs. Interestingly, a LOH on 16p11.2 was detected in all syndromic cases and 3 of 4 sporadic cases. This chromosomal

location contains genes such as *ZNF267, HERC2P4, TP53TG3D, LOC390705, TP53TG3, TP53TG3B, TP53TG3C, SLC6A10P, RNU6-76P, LINC00273, UBE2MP1, LOC283914, LOC146481, LOC100130700, RNA5SP411, and FLJ26245* not previously described in these cysts. A genetic alteration on 22q11.23 involving the genes *GSTTP1, LOC391322, GSTT1* and *GSTTP2* was detected in both cohorts of OKCs, with two syndromic cases showing gain of function mutations while in the sporadic cases one showed a gain and the other a loss. Furthermore, CNN-LOH on 2q22.1 was detected in both OKCs with *THSD7B* being the gene that is common to both. Finally, CNN-LOH on 3p21.31 was detected in one case each of syndromic (case 4) and sporadic (case 2) OKCs containing the genes *IP6K2, PRKAR2A, SLC25A20, ARIH2OS, ARIH2, P4HTM, WDR6, DALRD3, MIR425, NDUFAF3, MIR191, IMPDH2, QRICH1, QARS, USP19, LAMB2, LAMB2P1, CCDC71, KLHDC8B, LOC646498, CCDC36, C3orf62, MIR4271, USP4, GPX1, RHOA, TCTA, AMT, NICN1, DAG1, BSN-AS2, BSN, APEH, MST1, RNF123, AMIGO3, GMPPB, IP6K1, CDHR4, FAM212A, UBA7, MIR5193, TRAIP, CAMKV, MST1R, MON1A, RBM6, RBM5, RBM5-AS1, SEMA3F, GNAT1, SLC38A3, GNAI2, SEMA3B, LSMEM2, IFRD2, HYAL3, NAT6, HYAL1, HYAL2, TUSC2, RASSF1, ZMYND10, NPRL2, CYB561D2, TMEM115, CACNA2D2, C3orf18, HEMK1, CISH, MAPKAPK3, MIR4787, DOCK3, MANF, RBM15B* and *VPRBP*.

Discussion

The Molecular OncoScan® assay is a robust, well recognized cancer diagnostic microarray for the detection of CNVs, LOH, and cancer-related somatic mutations[24]. This array has the advantage of performing accurate molecular analysis on degraded DNA found in formalin-fixed paraffin-embedded (FFPE) tissues. Our study group previously used the OncoScan® assay to identify possible common genetic markers in oral cancer patients that could be of clinical significance[23]. More specifically, this array has successfully identified a new mutation (TP53:p.R213*:c.637C>T) in a non-smoking young adult with a poorly differentiated

keratinising squamous cell carcinoma of the tongue[25], and revealed CNAs and LOH in a rare case of HPV-negative oral squamous cell carcinoma with heterozygous p16 immunohistochemical expression[26].

In the case of OKCs, most molecular studies on non-syndromic single cysts and/or multiple syndromic cysts have been limited to the *PTCH* gene and a few selected genes of the Hedgehog pathway[8-10,16,17]. Limited studies exist on mutations in genes not related to the Hedgehog pathway[20]. Therefore, the current study utilised the OncoScan® assay to investigate whole genome CNAs, LOH and somatic mutations of cancer-associated genes in non-syndromic and syndromic OKCs. A molecular analysis of these two clinical cohorts based on their genetic alteration profiles was undertaken in search of common and differing genetic alterations.

In this study, most chromosomal alterations detected in both non-syndromic and syndromic OKCs were CNN-LOH. In most cases, these alterations were detected on unique chromosomal loci in the different patient groups. CNN-LOH alterations on 6p21.33 and 2q22.1 as well as a gain on 1p13.3 were detected in sporadic OKCs, but were absent in all syndromic cases. These may be molecular events useful for the differentiation of non-syndromic from syndromic OKCs. Although CNN-LOH alterations on the entire chromosome 9q were detected in both groups of OKCs, this alteration was present in all syndromic cases and three of the four non-syndromic cases. These findings corroborate previous reports where CNN-LOH alterations of the *PTCH* gene were detected in syndromic cases and a subset of non-syndromic OKCs[8,9,13,14].

The massive size of this alteration affecting an entire chromosomal arm may implicate several genes other than *PTCH*, such as *NOTCH1*, especially in the development of syndromic OKCs. Interestingly, alterations on 16p11.2 detected in all syndromic and three of four non-syndromic OKCs, have been previously detected in oral cancer patients[23]. However, none of the affected

genes (*ZNF267, HERC2P4, TP53TG3D, LOC390705, TP53TG3, TP53TG3B, TP53TG3C, SLC6A10P, RNU6-76P, LINC00273, UBE2MP1, LOC283914, LOC146481, LOC100130700, RNA5SP411 and FLJ26245*) in this region have been reported in any of these cases. Additionally, alterations on 22q11.23 detected in both non-syndromic and syndromic OKCs have also been detected in cases of oral cancer and their adjacent dysplastic epithelium[23]. Hence, the clinical relevance of the affected genes on these chromosomal loci (16p11.2 and 22q11.23) warrants further investigation.

This study confirmed mutations involving 9q22.3-q31 containing the *PTCH* gene, which is typical for syndromic[8,9] as well as in some non-syndromic OKCs [13,14]. In addition, a CNN-LOH on the entire 9q detected in all syndromic cases implicates other cancer-related genes located in this region, such as *NOTCH1*, which may be associated with the development of syndromic OKCs. This study found that CNN-LOH alterations on 6p21.33 and 2q22.1, as well as a gain on 1p13.3, were genetic alterations that may be useful for the differentiation of non-syndromic from syndromic OKCs. Finally, a LOH on 16p11.2 and CNN-LOH on the entire 9q detected in all syndromic cases and most non-syndromic cases could suggest the role of other genes in the development of both syndromic and non-syndromic OKCs.

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Conflict of Interests: Authors have no conflict of interest, financial or otherwise to declare

Ethics approval and consent to participate: This study was approved by the University of Pretoria Faculty of Health Sciences Research Ethics Committee (Protocol number 44/2010).

All participants gave written informed consent to participate in this study. This study was performed in accordance with the Declaration of Helsinki.

Author Contributions: W.F.P.vH. performed study concept and design; M.A.A., L.R., M.B.vH., M.S.P. and W.F.P.vH. performed development of methodology and writing, review and revision of the paper; M.A.A., L.R., M.B.vH., M.S.P. and W.F.P.vH. provided acquisition, analysis and interpretation of data. All authors read and approved the final paper.

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