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SPECIAL ANNOUNCEMENT

MANILA DECLARATION ON THE AVAILABILITY AND USE OF HEALTH RESEARCH INFORMATION IN AND FOR LOW- AND MIDDLE-INCOME COUNTRIES IN THE ASIA PACIFIC REGION

We, the participants in the Joint Meeting of the Asia Pacific Association of Medical Journal Editors (APAME), the Index Medicus of the South East Asia Region (IMSEAR), and the Western Pacific Region Index Medicus (WPRIM) held in Manila from 24 to 26 August 2015, in conjunction with the COHRED Global Forum on Research and Innovation for Health held in Manila from 24-27 August 2015, drawing on the Pre-Forum Discussions on HIFA from 20 July to 24 August 2015 *"Meeting the information needs of researchers and users of health research in low- and middle-income countries"* available at http://www.hifa2015.org/meeting-the-information-needs-of-researchers-and-users-of-health-research-2/ and the BMJ Blogs 20 July 2015 *"How can we improve the availability and use of health research in developing countries?"* available at http://blogs.bmj.com/bmj/2015/07/20/how-can-we-improve-the-availability-and-use-of-health-research-in-developing-countries/ :

CONSIDERING

That the WHO Constitution "enshrines the highest attainable standard of health as a fundamental right of every human being;" and that "The right to health includes access to timely, acceptable, and affordable healthcare of appropriate quality in tandem with "the underlying determinants of health," including" access to health-related education and information;"

That increasing the availability of quality health research information is fundamental to the successful attainment of global health and progressive realization of the right to health; and that all healthcare stakeholders (individuals, researchers, providers, professionals, leaders and policymakers) need seamless access to peer-reviewed research and information that are relevant to their respective contexts, and presented in a language they can understand;

That despite a growing momentum towards free and open access to research literature, and important initiatives, such as HINARI Access to Research In Health Programme and IRIS Institutional Repository for Information Sharing, that have helped to improve the availability of research in low- and middle-income countries, there continue to be many challenges, limitations and exclusions that prevent health research information from becoming freely and openly available to those who need it;

That the Global Health Library (GHL), Index Medicus of the South East Asia Region (IMSEAR), Western Pacific Region Index Medicus (WPRIM), and Asia Pacific Association of Medical Journal Editors (APAME)are important collaborative initiatives that can promote and uphold the availability and use of health research information especially in and for low- and middle-income countries in the Asia Pacific Region;

CONFIRM

Our commitment to champion and advocate for the increased availability, accessibility and visibility of health research information from and to low- and middle-income developing countries through our Journals, our respective National Associations of Medical Editors, and APAME;

Our commitment to make research information freely and openly available in the right language to producers and users of health research in low- and middle-income countries through IMSEAR, WPRIM, the Asia Pacific Medical Journal Articles Central Archives (APAMED Central) and other platforms;

Our commitment to improve availability, accessibility and interoperability of the different formats of health information suitable to different users in their respective contexts including through both conventional and alternative channels of research dissemination such as new and social media, mobile and disruptive technologies, blogging and microblogging tools and communities, and communities of practice;

CALL ON

Member States of and governments in the South East Asia and Western Pacific Regions, in collaboration with stakeholders from the non-government and private sectors to formulate and implement policies and certification schemes such as the COHRED Fairness Index™ (CFI) that promote free and open availability of health research information for both its producers and users, especially in low- and middle-income countries;

Stakeholders from the public and private sectors, national and international organizations, universities and academic societies, and discussion groups such as Healthcare Information for All (HIFA2015) to support IMSEAR, WPRIM, the GHL, APAMED Central, and develop Integrated Scholarly Information Systems and similar initiatives, in order to ensure the free, open and global accessibility of health research done in the South East Asia and Western Pacific Regions;

The Eastern Mediterranean Association of Medical Editors (EMAME), the Forum for African Medical Editors (FAME), the European Association of Science Editors (EASE), the World Association of Medical Editors (WAME), the International Committee of Medical Journal Editors (ICMJE), the Committee on Publication Ethics (COPE) and

other editors' and publishers' associations to support APAME in implementing various activities, guidelines and practices that would improve the quality, availability and accessibility of scientific writing and publications in the Asia Pacific Region and the world;

Bibliographic, Citation and Full-Text Databases such as PubMed, Global Health Database (CAB Direct), the Directory of Open Access Journals (DOAJ), EMBASE, SciELO Citation Index, Scopus, and the Web of Science to review their policies and processes for indexing Journals from low- and middle-income countries, as well as making health research information freely and openly available to users in these countries who cannot afford to pay for it;

COMMIT

Ourselves and our Journals to publishing innovative and solution-focused research in all healthcare and related fields such as health promotion, public health, medicine, nursing, dentistry, pharmacy, other health professions, health services and health systems, particularly health research applicable to low- and middle-income countries;

Ourselves and our publishers to disseminating scientific, healthcare and medical knowledge fairly and impartially by developing and using Bibliographic Indices, Citation Databases, Full-Text Databases and Open Data Systems including, but not limited to, such Regional Indexes of the Global Health Library as IMSEAR, WPRIM and APAMED Central;

Our organization, APAME, to building collaborative networks, convening meaningful conferences, and organizing participative events to educate and empower editors, peer reviewers, authors, librarians and publishers to achieve real impact, and not just impact factor, as we advance free and open access to health information and publication that improves global health-related quality of life.

26 August 2015, Manila

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This declaration was launched at the 2015 Convention of the Asia Pacific Association of Medical Journal Editors (APAME) held in Manila from 24 to 26 August 2015. It is concurrently published by Journals linked to APAME and listed in the Index Medicus of the South East Asia Region (IMSEAR) and the Western Pacific Region Index Medicus (WPRIM).It is co-published with special permission in the Pacific Journal of Medical Sciences that was represented in the APAME2015 Convention and Joint Meeting with the Western Pacific Region Index Medicus Regional Journal Selection Committee Meeting.

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INFORMATION

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ALLELIC FREQUENCIES OF P53 CODON 72 POLYMORPHISM AND HUMAN PAPILLOMAVIRUS-MEDIATED CERVICAL CANCER IN PAPUA NEW GUINEAN WOMEN

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ABSTRACT:
Cervical cancer is regarded as a sexually transmitted disease caused by the human papilloma virus (HPV) detected in up to 80 per cent of the cancer biopsies. Genetic susceptibility of a p53 allelic variant has been postulated to play a vital role in carcinogenesis. This study was aimed at determining the allelic frequencies of p53 codon 72 polymorphism in Papua New Guinean women and also assessing the presence of HPV in cervical cancer biopsies. Peripheral blood (3-5 mL) was collected from 53 healthy females of reproductive age (19-37 years) with no known past and current history of HPV infections. Sixty-two cervical biopsies along with cervical swaps were obtained from patients (19-54 years) with clinical symptoms and histopathological confirmation of cervical cancer. DNA was extracted from the peripheral blood samples and cervical samples. Exon 4 was amplified with PCR and further genotypic analyses performed by Restriction fragment length polymorphism (RFLP) and single-stranded conformational polymorphism (SSCP). Of the 53 normal samples analyzed, 3.8 % (2/53) were Arginine homozygous, 58.5 % were Proline homozygous and 37.7 % were heterozygous. For the cancer samples, 14.5 % (9/62) were Arginine homozygous, 54.8 % were Proline homozygous and 30.7% were heterozygous. HPV genome was detected in 83.9 % (52/62) of the cervical cancer samples. The genotypic trend and allelic frequencies were consistent with literature.

Keywords: p53 Codon 72 Polymorphism, Cervical Cancer, Human Papillomavirus
Submitted August 2015; Accepted September 2015

INTRODUCTION:

The wild-type *TP53* gene is polymorphic on Exon 4 codon 72, having a single nucleotide variation of either CGC or CCC coding for Arginine or Proline amino acid residue, respectively [1]. Allelic frequency distribution globally of exon 4 codon 72 has depicted the preference of Proline homozygotes predominantly around the equatorial regions and with the increase in latitude a transition to the Arginine variant [2]. The significance of the polymorphism stems from the increased

susceptibility to tumourigenesis associated with the Arginine homozygous genotype noted in various cancers including, cancers of the breast [3] bladder [4] , lung [5] , oropharynx [6] and the uterine cervix [7, 8]. It has been demonstrated that the polymorphic variants are functionally distinct, particularly in their ability to induce apoptosis, where the Arginine homozygous has an enhanced apoptotic potential, a property that can translate to cancer risk [9]. Furthermore, it is well documented that the p53 gene is mutated in over 50 percent of all cancers, demonstrating the vital role it plays in the maintenance of cellular integrity via its involvement in cell cycle regulation [10], DNA repair [11] and apoptosis [12].

The *Arg/Arg* genotype *versus Arg/Pro* or *Pro/Pro* genotypes at codon 72 of the p53 gene has been implicated as a risk marker in cervical neoplasia [13]. Cervical cancer is now regarded as a sexually transmitted disease based on the elucidation of human papillomavirus (HPV) oncoprotein E6 associated with premature ubiquitin-dependent proteolytic degradation of the p53 gene products [14] as the initial event in cellular transformation. The fact that only a fraction of females infected with HPV progress to cancer indicates that HPV infection is not a sufficient cause and points to co-factors. Genetic susceptibility is most likely to be one of the important co-factors. The high rate of occurrence of cervical cancer in certain ethnic groups also increases this suspicion. Genetic susceptibility is thought to play a crucial role in the initiation of cervical cancer. The implication of p53 gene and its allelic susceptibility to HPV mediated degradation serves as an appropriate starting point for the screening and detection of preceding events in cervical carcinogenesis. Studies have shown that the Arginine homozygous genotype of the p53 codon 72 polymorphism has being associated with a seven-fold increased susceptibility to HPV-mediated cervical cancer development [7].

In Papua New Guinea (PNG) there is no documentation of the allelic/genomic frequencies of the p53 polymorphism within the population. Since there is high incidence of cervical cancer cases in PNG the need to understand the genomic susceptibility is paramount. This study aimed to determine the allelic frequencies of p53 codon 72 polymorphisms in the Melanesian female population of PNG and assess its association with cervical cancer.

SUBJECTS AND METHODS:

Sample collection and DNA extraction

Peripheral blood (3-5 mL) was collected from 53 healthy females of reproductive age (19-37 years) with no known past and current history

of HPV infections. Sixty-two cervical biopsies along with cervical swaps were obtained from patients (19-54 years) with clinical symptoms of cervical cancer, which included post coital vaginal bleeding, offensive discharge and dyspareunia with further histopathological confirmation. The samples were stored at -20°C until DNA extraction.

DNA extraction was performed depending on the type of sample. DNA was isolated from peripheral blood lymphocytes by the standard phenol/chloroform/isoamyl alcohol method [15]. The biopsies were finely dissected with sterile blades prior to DNA extraction. The swaps were microcentrifuged and pellets collected and lysed with Lysis Buffer containing freshly thawed Proteinase K that was added and incubated at 55°C for 1 hour. The mixture was then heated to 100°C and cooled on ice, ready for polymerase chain reaction (PCR) and subsequent analysis.

Detection of p53 codon 72 polymorphisms by PCR-RFLP and PCR-SSCP

The p53 polymorphic region of exon 4 was PCR amplified from genomic DNA samples utilizing specific sense and antisense primers: p53F 5'-GCTCTTTTCACCCATCTACAG-3' and p53R 5'-AGGCATTGAAGTCTCATGGAAGC-3' (Operon). Each reaction mixture (50 µL) consisted of 1 µL genomic DNA template, 200 µM of each dNTP, 50 mM KCl, 10 µM Tris-HCl pH 8.5, 1.5 mM $MgCl_2$, 26 µM of each primer and 1.25 units *Taq* DNA polymerase (Promega). The mixture was heated to 95°C for 5 minutes then the amplification was carried out by 35 cycles of 30 seconds denaturation at 95°C, 60 seconds primer annealing at 62°C and synthesis at 72°C for 90 seconds. An additional 8 minutes of elongation was allowed at 72°C [15]. The PCR products were analyzed with appropriate DNA markers on a 1.5% agarose gel electrophoresis at 80 volts over 1 hour, stained with 10 mg/mL ethidium bromide and imaged on a UV light transilluminator to ensure correct length of DNA amplified for further analysis (Figure 1A).

PCR-RFLP:

Restriction fragment length polymorphism (RFLP) analysis of the PCR amplified DNA was performed in a reaction mixture (5 µL) containing 0.5 µL *Bst*U1, 0.5 µL 10X NE buffer (BioLabs) and 4 µL of amplified DNA. BstU1 cleaves a 5'-CGCG-3' sequence in the Arginine variant of codon 72. The mixture was incubated at 60°C for 16 hours, electrophoresed on a 1.5 % agarose gel at 80 volts, then stained with ethidium bromide solution and image using a UV transilluminator (Figure 1B) [15].

Figures 1 A, B and C: Determination of the allelic and genotypic frequencies of p53 codon 72 in normal and cancer patients

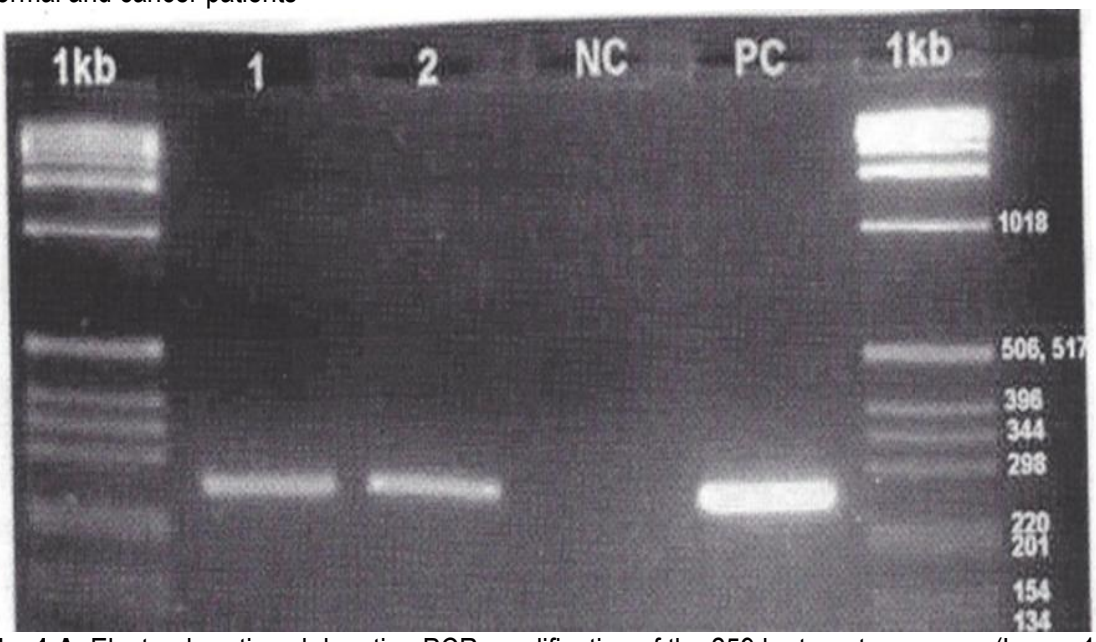

Fig. 1 A: Electrophoretic gel denoting PCR amplification of the 259 bp target sequence (Lanes 1 and 2), negative control (NC) and positive control (PC).

Fig. 1 B: RFLP analysis utilizing *Bst*U1 digestion of PCR amplified p53 product to demonstrate codon 72 polymorphism of homozygous Arginine or Proline or their heterozygous genotype.

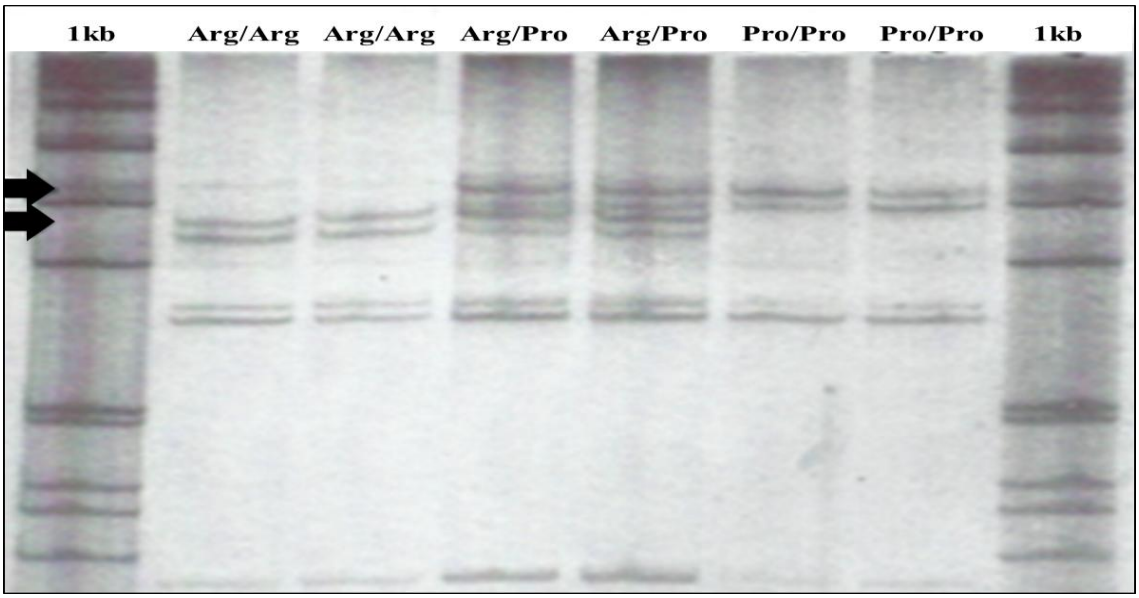

Fig. 1 C: SSCP analysis of same samples in B using PAGE and silver staining.

PCR-SSCP

The single-stranded conformational polymorphism (SSCP) bands were obtained by denaturing a sample mixture (10 µL) of 5 µL PCR amplified DNA and 5 µL of 90 % formamide at 100˚C for 4 minutes then immediately chilling on ice prior to running on a 8 % polyacrylamide gel electrophoresis (PAGE). This was then silver stained by fixing in 40 % ethanol for 30 minutes, then in 10 % ethanol for 30 minutes followed by soaking in impregnation solution (11 mM $AgNO_3$ in 20 mL $_{dd}H_20$) for 30 minutes and subsequently in developing solution (7.5g NaOH, 2 mL of 36 % formaldehyde in 250 mL of $_{dd}H_20$) until the SSCP bands discerned then immersed in 5 % acetic acid to stop reaction. The gels were then image on a UV transilluminator gel documentation system (Figure 1C) [16].

Detection and typing of HPV

The presence of HPV DNA was detected by nested PCR utilizing general primers GP5+ and GP6+ as initial primers and MY09 and MY11 as subsequent primers [17]. HPV type specific PCR for HPV-16 and HPV-18 were done utilizing the sense and antisense primers HPV16F/R and HPV18F/R respectively (Table 1). The extracted DNA were first amplified for detection by general primers in a reaction mixture (50 µL) consisting of 1.25 mM of each dNTP, 10 µM of GP5+ and GP6+ (primary) and later MY09 and MY11 (secondary), 35 mM of $MgCl_2$ in 10x PCR buffer and 0.2 µL *Taq* polymerase. The mixture was heated for 1 minute at 95°C prior to subjecting to 30 cycles

of primary PCR amplification at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 60 seconds followed by elongation at 72°C for 7 minutes.

Table 1: The HPV forward and reverse primers of both the general and type-specific sequences for nested-PCR [17].

Primer	Sequences (5'→3')
	Consensus primers for HPV L1 region
MY09	CGTCCAAGAGGATACTGATC
MY11	GCACAGGGTCATAATAATGG
GP5+	TTTGTTACTGTGGTAGATACTAC
GP6+	GAAAAATAAACTGTAAATCATATTC
	Types specific HPV primers
HPV16F	ACCGAAACCGGTTAGTATAAA
HPV16R	GATCAGTTGTCTCTGGTTGCAAAT
HPV18F	CACACCACAATACTATGGCGCGCT
HPV18R	CTGCTGGATTCAACGGTTTCTGGC

Secondary amplification involved preheating at 95°C for 1 minutes then 30 cycles at 94°C for 30 seconds, 45°C for 30 seconds and 72°C for 30 seconds and ending with elongation period for 7 minutes at 72°C. The PCR products were analyzed as previous stated. For HPV type specific detection the secondary amplification step involved type specific primers for the high risk HPV (hr HPV) subtypes investigated (Figure 2A).

RESULTS:

The study involved a total of 115 subjects; 62 histologically confirmed cervical cancer patients obtained from the Goroka Base Hospital and 53 healthy females from the School of Medicine and Health Sciences (SMHS), University of Papua New Guinea (UPNG). The types of cancers included pre-neoplastic lesions CIN I-III, squamous cell carcinoma and adenocarcinoma. Two highly sensitive tests were utilized to detect and confirm the genomic composition of the samples. RFLP and SSCP analysis followed the amplification of a 259 base pair (bp) sequence containing the codon 72 polymorphism which is a part of exon 4.

HPV association in cervical cancer samples

HPV was detected in 52 out of 62 (83.9 %) cervical cancer samples of which eight out of

the total HPV infected samples showed co-infection, while 10 samples were negative (Figure 2B). The high risk HPV types screened included HPV-6, 11, 16, 18, 31, 33 and 35. Type-specific determination of the high risk types showed HPV-11 accounting for 26.9 %, HPV-16 – 19.2 %, HPV-18 – 19.2 % and HPV-33 – 3.9 % while other HPV types accounted for the remaining 30.8% (Figure 2B and insert). HPV-6, -31 and -35 were not detected.

Figure 2. The general detection HPV and of high-risk type HPV in cervical cancer specimen

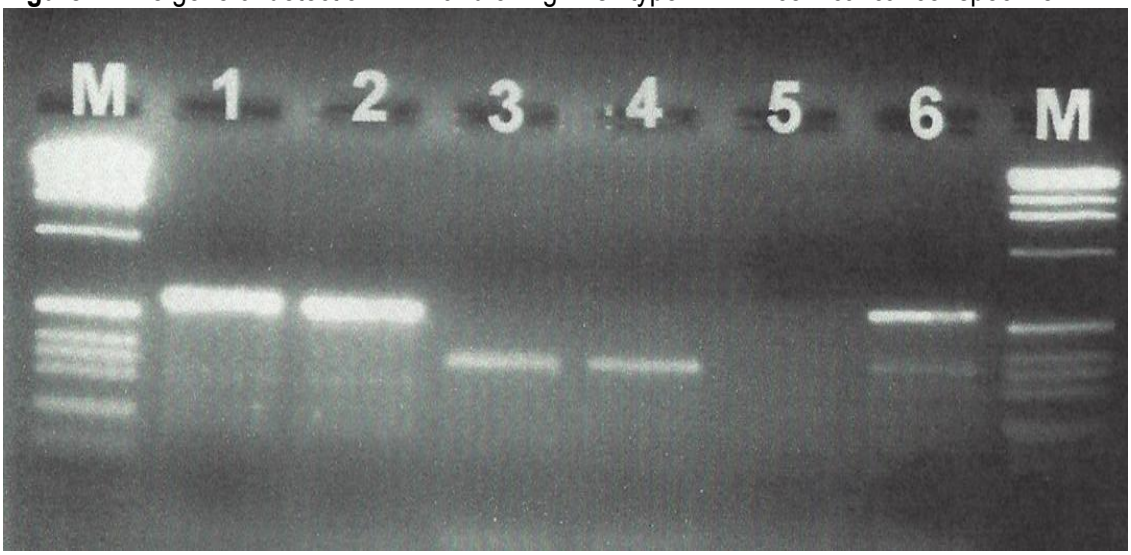

Fig. 2 A: Electrophoretic gel showing PCR amplification of HPV-16 (Lane 1 and 2), HPV-18 (Lanes 3 and 4), negative control (Lane 5), positive control for both HPV 16 and -18 (Lane 6) and 1 kb DNA ladder (M).

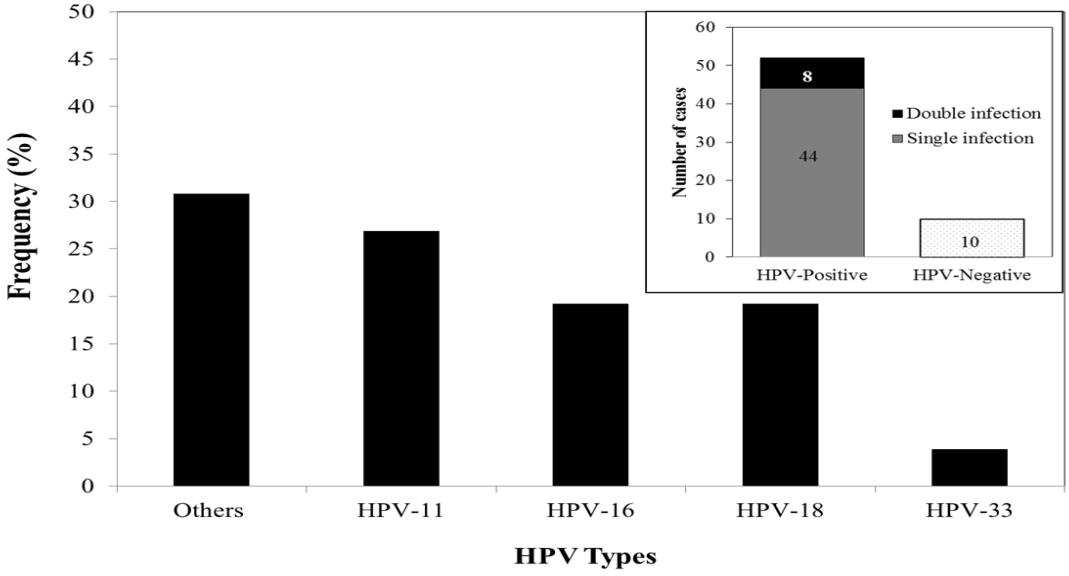

Fig. 2 B: The frequency of HPV subtypes in the population studies. (**B insert**) Represents the total number of HPV positive and negative cases and also includes cases of double infection.

p53 Codon 72 allelic frequencies

The allelic frequencies were calculated using the Hardy-Weinberg equation ($p^2 + 2pq + q^2 = 1$), where frequency of genotypes *Arg/Arg* = p^2, *Arg/Pro* = 2pq and *Pro/Pro* = q^2. On the other hand, looking at the results separately, the 53 normal subjects showed genotypic frequencies of *Arg/Arg* as 3.8 %, *Arg/Pro* represented 37.7 % and Pro/Pro 58.5 %, while, on the other hand, genotypic frequencies of the 62 cancer samples showed *Arg/Arg* genotype as 14.5 %, *Arg/Pro* represented 30.7% and *Pro/Pro* 54.8 % (Figure 3).

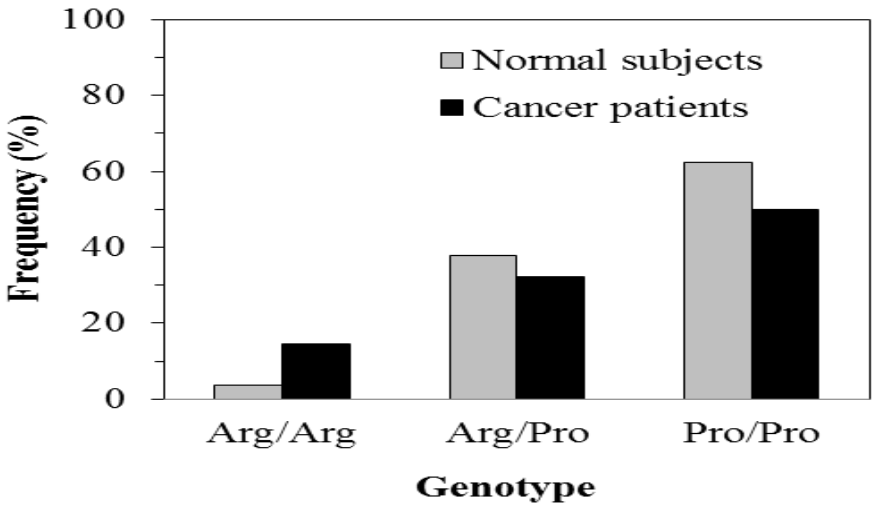

Fig. 3: Graph showing genotypic frequency distribution of the p53 codon 72 polymorphism in the studied population.

DISCUSSION:

It has been postulated that the frequencies of p53 codon 72 genotypes vary according to different ethnic groups [8, 18]. On the contrary, the polymorphic allelic frequency distribution of Arginine and Proline has demonstrated a distinct geographical pattern where the prevalence of the Proline allele across multiple genetically unrelated populations increases as one nears the equator. An impression that the Proline allele confers a protective effect against certain forms of UV damage [19]. The findings in this study are consistent with this observation revealing a higher frequency of the Proline allele (77.4 % and 70.2 % for normal and cancer subjects, respectively (Table 2)) in the Melanesian population. On the other hand, the frequencies of the p53 Arginine alleles are

generally lower around the equatorial region and increases with latitude, which was also noted in this study. The relationship between the allelic and genotypic susceptibility of the p53 codon 72 polymorphism and cervical cancer development could not be elucidated due to the lack of statistical power, despite the *Arg/Arg* homozygous genotype been established as the susceptible genotype. The trend observed in this study leans towards this association as the frequency of the patients with *Arg/Arg* genotype (9/62 – 14.5 %; Table 2) was relatively high for a small sample size. As expected the frequency of the *Pro/Pro* genotype was high in both the normal and the cancer samples. The observed heterozygote (*Arg/Pro*) distribution of the patients was slightly lower than that of the control population; an observation that favours the 'loss of heterozygosity' theory preferred for HPV mediated p53 degradation.

Table 2: The frequency distribution of p53 codon 72 and the HPV status of cervical cancer patients in Papua New Guinean women

Genotype frequencies

Genotype	**Normal (%)**	**Patients (%)**
Arg/Arg	2/53 (3.8)	9/62 (14.5)
Arg/Pro	20/53 (37.7)	19/62 (30.7)
Pro/Pro	31/53 (58.5)	34/62 (54.8)

Allelic frequencies

Allele	Frequencies (%)
P 53 Arginine	29.8
P 53 Proline	70.2

The introduction of the bivalent HPV vaccine [20] in recent years is a major breakthrough in the proactive management of cervical cancer. However, in developing countries like PNG where vaccination has not yet being routinely administered and thus herd immunity is yet to be achieved, cervical cancer is still a major problem. As such, the battle against this preventable and curable disease is still raging in all fronts. The basic approach of Papaniculou (PAP) smear done routinely along with adequate education on early symptom recognition and clinical presentation is still the gold standard of management, but again coverage is minimal.

HPV infection and persistence are necessary for the initiation and progression of cervical cancer [8, 21]. The HPV prevalence in PNG in general is not known but a small study done at the PNG Institute of Medical Research (PNGIMR) of 114 females attending the Gynaecology clinic at the Goroka Base hospital revealed a 52 % overall incidence with high-risk types HPV-16 and 18 accounting for 33 % [22]. In this study, 83.9 % (52/62) of cervical cancer samples analyzed contained HPV genome (Table 3), a finding that is consistent with literatures which have shown detection in up to 99.7 % of cases [23].

Table 3: HPV status of cervical cancer patients in Papua New Guinea

General detection	HPV-11	HPV-16	HPV-18	HPV-33	Others
52/62 (83.9 %)	14/52 (26.9 %)	10/52 (19.2 %)	10/52 (19.2 %)	2/52 (3.9 %)	16/62 (30.8 %)

The high risk types HPV-16 and 18 together accounted for 38.4 % of all cervical cancer cases while HPV-11 genome was detected in 26.9 %. Furthermore, the presence of co-infection of these high risk HPV types was detected in 15.38 % (8/62), a potential scenario where cancer initiation and progression is likely to be rapid. The data shows females as young as 19 years developing HPV-positive cervical cancer. This further adds to the fact that the HPV oncoproteins not only degrade p53 gene (E6) but the E6-E7 fusion protein degrades RB1 genes [24], both proteins that play vital roles in the maintenance of cellular genome integrity.

CONCLUSIONS:

The allelic and genotypic frequency distribution of the p53 codon 72 polymorphism in the Papua New Guinean population is shown to incline towards the general global occurrence. The results obtained in this study provide a glimpse of the amount of work required to establish the extent of the HPV-mediated cervical cancer in PNG, starting with the determination of the incidence and prevalence of high-risk HPV types within the population. This may lead to the recommendation for compulsory vaccination schedules to be incorporated into the national immunization programs. And finally, the high incidence of

HPV in cervical cancer specimen highlights the rationale for HPV testing in addition to cervical cytology in routine cervical screening.

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REFERENCES

1. McGregor, J.M. Harwood, C. A., Brooks, L., Fisher, S. A., Kelly, D. A. O'nions, J. , Young, A. R., Surentheran, T., Breuer, J. and Millard, T. P., *Relationship between p53 codon 72 polymorphism and susceptibility to sunburn and skin cancer.* Journal of investigative dermatology, 2002. 119(1): p. 84-90.
2. Beckman G, B.R., SjÃnlander A, Saha N, Holmberg PÃ, KivelÃn A, Beckman L, *Is p53 polymorphism maintained by natural selection?* Human heredity, 1994. 44(5): p. 266-270.
3. Papadakis, E., D. Dokianakis, and D. Spandidos, *p53 codon 72 polymorphism as a risk factor in the development of breast cancer.* Molecular Cell Biology Research Communications, 2000.3(6):p.389-392.
4. Soulitzis, N., Sourvinos, G.,Dokianakis, D. N.,Spandidos, D. A., *p53 codon 72 polymorphism and its association with bladder cancer.* Cancer letters, 2002. 179(2): p. 175-183.
5. Fan, R., Wu, M-T., Miller, D., Wain, J. C., Kelsey, K. T.,Wiencke, J.K. and Christiani, D. C., *The p53 codon 72 polymorphism and lung cancer risk.* Cancer Epidemiology Biomarkers & Prevention, 2000. 9(10): p. 1037-1042.
6. Kietthubthew, S., Sriplung, H. Au, W. W. and Ishida, T., *The p53 codon 72 polymorphism and risk of oral cancer in Southern Thailand.* Asian Pacific Journal of Cancer Prevention, 2003. 4(3): p. 209-214.
7. Storey A, T.M., Kalita A, Harwood C, Gardiol D, Mantovani F, Breuer J, Leigh I M, Matlashewski G and Banks L, *Role of a p53 polymorphism in the development of human papilloma-virus-associated cancer.* Nature, 1998. 393(6682): p. 229-234.
8. Rosenthal, A.N., Ryan, Andy., Al-Jehani, Rajai M., Storey, Alan., Harwood, Catherine A and Jacobs, Ian J, *p53 codon 72 polymorphism and risk of cervical cancer in UK.* The Lancet, 1998. 352(9131): p. 871-872.
9. Dumont, P., Leu, J-J., Della Pietra, A. C., George, D. L. and Murphy, M., *The codon 72 polymorphic variants of p53 have markedly different apoptotic potential.* Nature genetics, 2003. 33(3): p. 357-365.
10. Martinez, J., I. Georgoff, and A. Levine, *Cellular localization and cell cycle regulation by a temperature-sensitive p53 protein.* Genes & development, 1991. 5(2): p. 151-159.
11. Smith, M.L., Chen, I-T., Zhan, Q., O'Connor, P. M. and Fornace Jr, A. J., *Involvement of the p53 tumor suppressor in repair of uv-type DNA damage.* Oncogene, 1995. 10(6): p. 1053-1059.

12. Speidel, D., *Transcription-independent p53 apoptosis: an alternative route to death.* Trends in cell biology, 2010. 20(1): p. 14-24.
13. Koushik, A., R.W. Platt, and E.L. Franco, *p53 Codon 72 Polymorphism and Cervical Neoplasia A Meta-Analysis Review.* Cancer Epidemiology Biomarkers & Prevention, 2004. 13(1): p. 11-22
14. Scheffner, M., Werness, B. A., Huibregtse, J. M., Levine, A. J. and Howley, P. M., *The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53.* Cell, 1990. 63(6): p. 1129-1136.
15. Sujirachato, K., Chiewsilp, P., Tsuji, K., Inoko, H., Panyim, S., Mongkolsuk, T. and Leetrakool, N., *HLA class II polymorphism in Thais detected by PCR-SSO and PCR-RFLP.* Tissue antigens, 1994. 43(4): p. 224-228.
16. Hayashi, K., *PCR-SSCP: a method for detection of mutations.* Genetic Analysis: Biomolecular Engineering, 1992. 9(3): p. 73-79
17. Zehbe, I. and E. Wilander, *Two consensus primer systems and nested polymerase chain reaction for human papillomavirus detection in cervical biopsies: a study of sensitivity.* Human pathology, 1996. 27(8): p. 812-815.
18. Thomas, M., Kalita, Ann., Labrecque, Sylvie., Pim, David., Banks, Lawrence and Matlashewski, Greg, *Two polymorphic variants of wild-type p53 differ biochemically and biologically.* Molecular and cellular biology, 1999. 19(2): p. 1092-1100.
19. Thomas, M.C., Jost, Christine A., Brooks, Louise A., Irwin, Meredith S., O'Nions, Jenny., Tidy, John A., James, Nick., McGregor, Jane M., Harwood, Catherine A and Yulug, Isik G, *A common polymorphism acts as an intragenic modifier of mutant p53 behaviour.* Nature genetics, 2000. 25(1): p. 47-54.
20. Stanley, M., D.R. Lowy, and I. Frazer, *Prophylactic HPV vaccines: underlying mechanisms.* Vaccine, 2006. 24: p. S106-S113.
21. Hayes, V.M., Hofstra, Robert MW., Buys, Charles HCM., Hollema, Harry., van der Zee and Ate GJ, *Homozygous arginine-72 in wild type p53 and risk of cervical cancer.* The Lancet, 1998. 352(9142): p. 1756.
22. Mgone, C., *Is uterine cervical cancer a potentially preventable sexually transmitted disease?* Papua New Guinea Medical Journal, 1998. 41(2): p. 47-50.
23. Walboomers, J.M., et al., *Human papillomavirus is a necessary cause of invasive cervical cancer worldwide.* The Journal of pathology, 1999. 189(1): p. 12-19.
24. Scheffner, M., Manger, K., Huibregtse, J. M. and Howley, P. M., *Targeted degradation of the retinoblastoma protein by human papillomavirus E7-E6 fusion proteins.* The EMBO journal, 1992. 11(7): p. 2425.

ANTIBACTERIAL ACTIVITY FROM CO-CULTURE OF A PAPUA NEW GUINEA FUNGAL ENDOPHYTE WITH *BACILLUS SUBTILIS*

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ABSTRACT:

Fungal endophytes are fungi that reside in tissues of healthy living plants offering chemical protection and promoting growth of host plants. The chemistry and biological properties of their secondary metabolites from monoaxenic cultures have been extensively studied, but co-culture techniques using endophytes remain largely unexplored. Co-culture of bacteria with endophytic fungi have shown to be effective, leading to the discovery of novel chemistry while simultaneously addressing the problem of rediscovery of known metabolites from the monoaxenic culture techniques. Forty microliters (40µL) of culture broth consisting of monoaxenic and co-cultures were placed in separate agar wells with 5µg ciprofloxacin as positive control. The co-culture broth from one unidentified endophytic fungus with *B. subtilis* showed a marked zone of inhibition measuring 24.7 ±0.6 mm in comparison to ≥21.0 mm as the sensitivity range for ciprofloxacin against *Escherichia coli* while its monoaxenic culture exhibited no inhibition, but furthermore showed moderate activity (16.7 ± 1.2 mm) against *B. subtilis.* The isolation and chemical characterization of the active component and the mechanism of *B.subtilis* induction is under investigation. It is hoped that this unique Papua New Guinea endophyte will provide broad-spectrum antibiotic to combat the growing global problem of drug resistant infections.

KEYWORDS: Monoaxenic culture, Co-culture, Fungal endophyte, Lianas, Antimicrobial activity

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INTRODUCTION:

Fungal endophytes are fungi that thrive in tissues of virtually all healthy plants for most of their lives. They offer chemically-mediated host protection and resistance to external stress. The chemistry and biological properties of fungal endophytes have been extensively studied in the search for more effective anti-infective agents to combat emerging drug-resistant infectious diseases [1-3].

Co-culture technique is a simple method of culturing two or more microbial strains. This method has shown to effectively enhance chemical yield of present constituents while simultaneously yielding novel defensive chemicals. Molecular studies have demonstrated that genes involved in the biosynthesis of antibiotics by microbes remain silent under classical monoaxenic culture but become activated when whole cell organisms of two or more different strains are cultured together [4-7].

The fungal endophytes in foliar assemblages in Papua New Guinea remain largely understudied and can offer potential lead molecules for drug discovery. The current study was carried out to determine the antibacterial activity of defensive chemicals produced by fungal endophytes isolated from an unknown endemic PNG liana species under monoaxenic and co-culture conditions.

METHODOLOGY:

Sampling

Foliar samples from a mature healthy liana species were randomly collected (diameter, breadth and height (dbh) of ≥10cm at 1.5-2.0m above ground) at Varirata National Park outside of Port Moresby, National Capital District [8-10]. The fresh foliar samples were kept in a cool storage container and transported to the drug discovery laboratory in the School of Medicine and Health Sciences, University of PNG to initiate work on fungal endophytes. Voucher specimens of the host plants were prepared and deposited at the Biological Sciences Herbarium, UPNG.

Surface-sterilization

Surface-sterilization reagents were used to eliminate epiphytes from the foliar samples, and carried out within 48 hours after sampling in field. Methods used were adapted from three different studies [11-13]. Briefly, whole foliar samples were held under running tap water for about 10 minutes to wash out any dirt and debris. Afterwards, a 2mm x 2mm incision were made by sterile surgical blade (size 10) and the cuts dipped sequentially in 70% ethanol (EtOH) for 1 minute, then in 2.6% sodium hypochlorite (NaOCl) for 3minutes and finally twice in sterile double distilled water.

Pure culture

Sterile potato dextrose agar (PDA) supplemented with chloramphenicol (100mg/ 1L of PDA) was used for the isolation and promotion of fungal endophytes while suppressing other forms of endophytes [14-16]. To determine the effectiveness of this surface-sterilization method, aseptically air-dried sterile cuts were printed on control plates that showed absence of growth on control plates [17].

The cuts were left on plates for 3-4 weeks at 25°C, encouraging the outgrowths of fungal endophytes. Contaminations were noticeable as growths protruding from the outer margins of plates and thus discarded. Pure (single) fungal isolates were achieved by continuous sub-culture of growths on freshly made PDA with an incubation period of 3-4 weeks per sub-culture [18]. Active stock cultures were maintained on nutrient agar (NA) and served as the primary source for small-scale fermentation.

Small- scale monoaxenic culture

Agar plugs (5mm x 5mm) were aseptically transferred from the active stock cultures into 100ml of freshly prepared sterile nutrient broth (3.0g beef extract, 5.0g peptone per 1L) in sterile 500ml Erlenmeyer flasks. The fungal isolates were incubated for 3-4 weeks in stationary phase at 25 °C with periodic shaking. Controls of cell-free nutrient broth were used to monitor contamination [2, 19].

Small- scale co-culture with *B. subtilis*

Co-cultures of individual isolates with *B. subtilis* were prepared at the same time as their mono-cultures [20]. Sterile nutrient broth (100ml) was inoculated with single fungal isolates as per the above method. A 24 hour colony of *B. subtilis* grown on Mueller Hinton Agar (MHA) at 37°C was aseptically added into the nutrient broth. The co-culture flasks were left standing at 25 °C for 2 weeks with periodic shaking [2].

Concentration of culture broth

Both culture broths (100ml) were aseptically transferred to new sterile 50ml tubes and centrifuged at 10 000 rpm and 4°C for 10 minutes. The supernatant collected (100ml) were reduced to 3ml through vacuum drying using an Eppendorf concentrator set at 4000rpm and 30°C. Concentrated supernatant were maintained at 4°C for future work [16].

Preliminary Bioassay

Antimicrobial agar-well diffusion assay was carried out in triplicates using supernatants from both the monoaxenic and co-cultures. Test pathogens employed include *Staphyloccocus aureus* ATCC 25923, *B. subtilis* ATCC 6633, *Escherichia coli* ATCC 25922 and *Pseudomonas aerigunosa* ATTC 27853. The standards of Clinical and Laboratory Standards Institute (CLSI) were followed [21]. Briefly, bacterial inoculum matching standard 0.5Mc Farland turbidity was prepared and subsequently swabbed onto

sterile and freshly premade MHA to produce a uniform bacterial lawn. Wells were then aseptically dug using the base of sterile Pasteur pipettes (6mm in diameter) and the supernatant (40µL) added thereafter. Ciprofloxacin which is a broad-spectrum antibacterial agent was employed as positive control. A standard dose of ciprofloxacin (5µg) was obtained through serial dilution in a 96-well plate and transferred onto the pre-dug wells. The plates were placed in incubator at 37.5°C for 24 hours and the zone of inhibition (in mm) measured afterwards.

RESULTS:

The four fungal isolates that were used in this experimental set-up were HK060 3P2GB1A, HK060 2P3C2A, HK060 3P3C1 and HK060 2P3D1A (Figure 1). The antibacterial activity of co-culture 2P3C2A against *E. coli* and *B. subtilis* were significant relative to their monoaxenic culture when grown in nutrient broth showing bacterial sensitivity at 24.7 ± 0.6 mm and 16.7±1.2mm respectively (Tables 1 & 2, and Figure 2). The monoaxenic culture of isolate 3P2GB1A demonstrated a significant zone of inhibition against *E. coli,* measuring 22.0± 1.0 mm comparable to the sensitivity range of ciprofloxacin at ≥ 21.0 mm according to CLSI (Table 1). Antibacterial activity was observed for *E. coli* and *B. subtilis* and hence only the antibacterial results against these 2 bacterial test pathogens were presented in Tables 1 and 2.

Table 1: Antibacterial activity against *E.coli*

		Zone of inhibition (mm)		
	Fungal isolate	Co-culture*	Pure culture¶	Positive control§
1	2P3C2A	24.7±0.6	6.0±0.0#	37.3±0.6
2	3P2GB1A	6.0±0.0	22.0±1.0	34.7±0.6
3	3P3C1	15.0±0.0	6.0±0.0	38.0±0.0
4	2P3D1A	6.0±0.0	6.0±0.0	38.0±0.0

Keys: *Co-culture with *B. subtilis*, § Ciprofloxacin, ¶Pure culture without *B. subtilis*, #6mm represents the diameter of the agar well

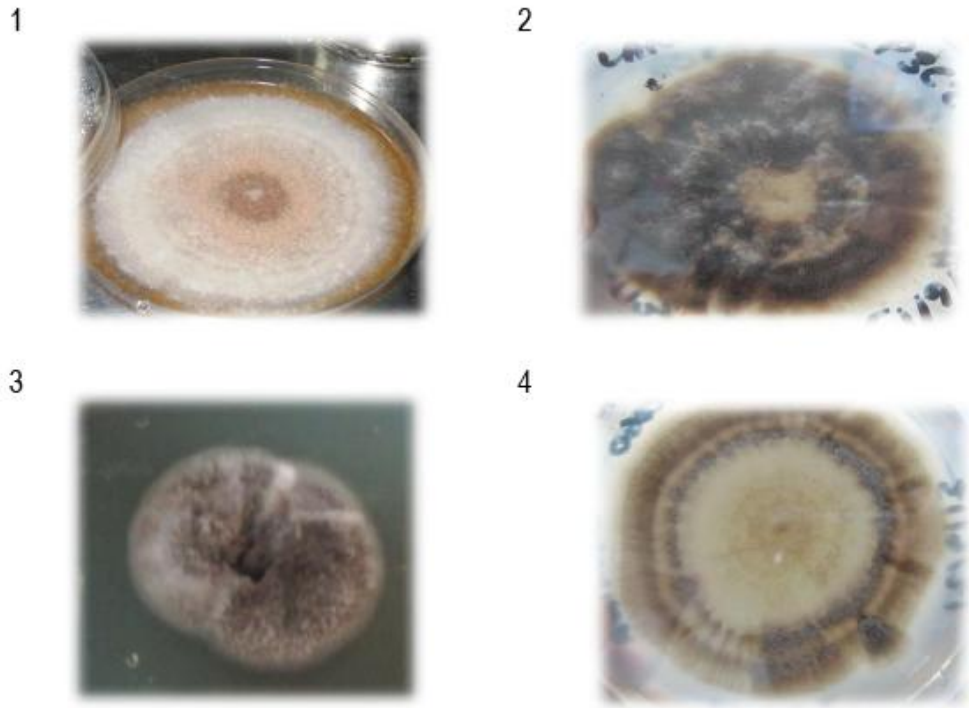

Figure 1: Fungal endophytes: (1- 3P2GB1A 2;(2-2P3C2A);(3- 3P3C1);(4- 2P3D1A)

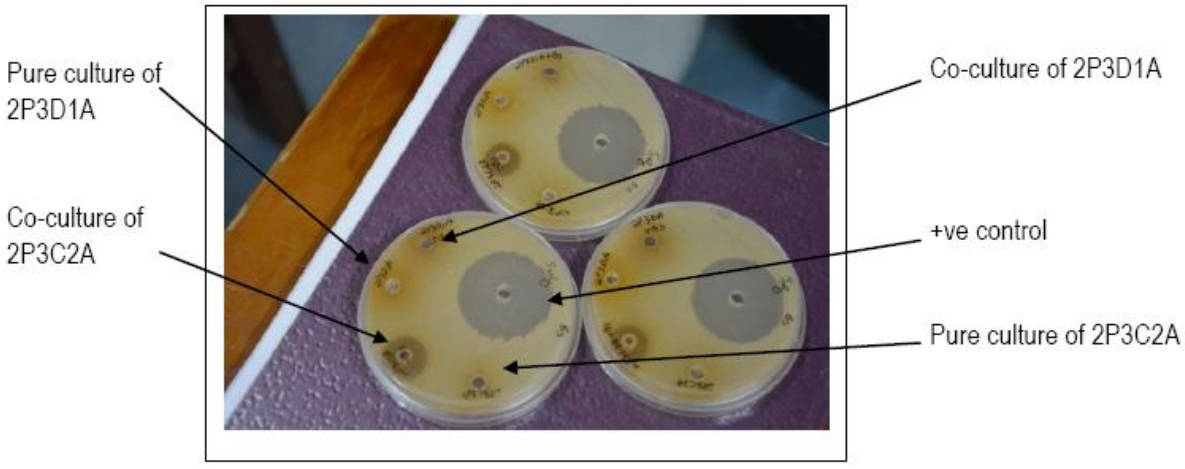

Figure 2: Antibacterial activity of monoaxenic culture and co-culture of 2P3C2A and 2P3D1A against *B. subtilis* shown in triplicates

Table 2: Antibacterial activity against *B.subtilis*

		Zone of Inhibition (mm)		
	Fungal isolate	**Co-culture***	**Pure culture¶**	**Positive control§**
1	2P3C2A	16.7±1.2	6.0±0.0#	36.7±0.0
2	3P2GB1A	6.0±0.0	6.0±1.0	37.3±0.6
3	3P3C1	13.0±0.0	6.0±0.0	38.3±0.6
4	2P3D1A	6.0±0.0	6.0±0.0	38.3±0.3

Keys: *Co-culture with *B. subtilis*, § Ciprofloxacin, ¶Pure culture without *B. subtilis*, #6mm represents the diameter of the agar well

DISCUSSION:

The four fungal endophytes used in this study, have been isolated from the foliar samples of a liana species HK060 (yet to be taxonomically verified). When grown in monoaxenic culture, the fungi 3P2GB1A isolated from midrib segment of HK060, demonstrated significant antibacterial activity against gam-negative *E.coli,* with a zone of inhibition measuring 22.0± 1.0 mm. Extensive reviews have been published describing the diverse chemical and biological properties of fungal endophytes [1,3,12]. Using molecular sequence data of 1403 endophytic strains, Arnold E and Lutzoni F have shown that incidence of foliar endophytes on a latitudinal basis, increases from Canadian arctic to the equatorial zone with less than 1 % to 99% in tissue segments respectively [22]. These findings infer that tropical foliar assemblages are a biodiversity hotspots for rare species of fungal endophytes with unknown host ranges and unrealized biological and chemical potential.

The cell-free supernatant from co-culture 2P3C2A (yet unknown) and bacteria *B. subtilis* exhibited a marked zone of inhibition (24.7 ± -0.6 mm) against *E. coli* when compared to its corresponding monoaxenic culture. When tested against *B. subtilis*, the same co-culture exhibited the greatest zone of inhibition (16.7 ± 1.2 mm). The cell-free supernatant from co-culture 3P3C1 with *B. subtilis* exhibited a zone of inhibition measuring 15.0± 0.0 mm and 13.0mm ±0.0mm against gram-negative bacteria *E.coli* and gram-positive bacteria *B. subtilis* respectively. The marked zone of inhibition demonstrated through co-cultures in this study, may support past studies of bacteria- fungi co-cultures and the discovery of

novel chemical compounds and or accumulation of presently constitutive compounds. *In vitro* culture of endophytic fungi with phytopathogens (bacteria or fungi) has led to the production of previously unsurfaced defensive chemicals. For instance, the co-culture of endophytic fungi *Fusarium tricinctum* with *B. subtilis* strain 168 trpC2 on solid rice medium led to 78-fold increase in the accumulation of constitutively present chemical compounds and spectral analysis identified 3 additional novel compounds namely macrocarpon C, *N*- (carboxymethyl)-anthranilic acid and (-) – citreoisocoumarinol. No such effect was observed with its monoaxenic control [20].

The cell-free supernatant from co-culture of unknown fungi 2P3C2A with *B. subtilis* exhibited significant zone of inhibition compared to its monoaxenic culture. The absence of zone of inhibition observed with the same supernatant against test pathogens *S. aureus* and *P. aerigunosa* may suggest specificity of interaction between the unknown fungi 2P3C2A and *B. subtilis* giving rise to the biosynthesis of defensive chemicals to which only *E. coli* was most sensitive to and to a lesser extent *B. subtilis*. Furthermore, the monoaxenic culture of the unknown endophytic fungi 3P2GB1A exhibited a pronounced zone of inhibition (22.0± 1.0 mm) against *E. coli.* Schroeckh V and colleagues demonstrated that specific intimate physical interaction between whole cell organisms was required to induce the biosynthesis of the dormant archetypal polyketide orsellinic acid (OA) and its derivative lecaronic acid. From 58 streptomycete species, *Streptomycete rapamycinicus* solely triggered the induction of OA in *Aspergillus nidulans* [7].

CONCLUSION:

The results of the study showed that co-culture technique greatly enhanced the antibacterial activity especially of the unknown fungal isolate 2P3C2A when cultured with *B. subtilis*. It also provided insight into antibacterial property of unknown fungal isolate 3P2GB1A. The isolation and chemical characterization of the active component from the co-culture of 2P3C2A with *B. subtilis* and the mechanism of *B. subtilis* induction are currently under investigation. It is hoped that this unique Papua New Guinea endophyte will provide a novel antibiotic to combat the growing global problem of drug resistant infections.

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REFERENCES:

1. Strobel G, Daisy B, Castillo U and Harper J. Natural Products from

Endophytic Microorganisms. J. Nat. Prod.2004, 67(2): 257-268.

2. Tayung K, Barik P, Jha K and Deka C. Identification and Characterization of antimicrobial metabolite from an endophytic fungus, Fusarium solani isolated from bark of Himalayan yew. Mycosphere. 2011 2 (3): 203-2013.
3. Joseph B and Priya M. Bioactive compounds from endophytes and their potential in Pharmaceutical Effect: A Review. American Journal of Biochemistry and Molecular Biology.2011, 1 (3): 291-309.
4. Ho W, To P and Hyde K. Induction of antibiotic production of freshwater fungi using mix-culture fermention. Fungal Diversity.2003, 12: 45-51.
5. Fox E and Howlett B. Secondary metabolism: regulation and role in fungal biology. Current Opinion in Mycrobiology. 2008: 481-487.
6. Ochi K and Hosaka T. New strategies for drug discovery: activation of silent or weakly expressed microbial gene clusters. Appl. Microbiol Biotechnol. 2013,97: 87-98.
7. Schroeckh V, Scherlach K, Nützmann H, Shelest E, Schmidt Heck W, Schuemann J, Martin K, Hertweck C and Brakhage A. Intimate bacterial-fungal interaction triggers biosynthesis of archetypal polyketides in Aspergillus nidulans. PNAS. 2009,106 (34):14558-14563.
8. Lü X, Tang J, Feng Z and Li M. 2008. Diversity and above ground biomass of lianas in the tropical seasonal rainforests of Xizhuangbanna, SW China. Int. J.Trop.Biol.2008, 57(1-2):211-222.
9. Schnitzer S and Bongers F. The ecology of lianas and their role in forests. Trends in Ecology and Evolution.2002, 17 (5): 223-230.
10. Appanah S, Gentry A and LaFrankie J. 1992. Liana diversity and species richness of Malaysian rainforests. Journal of Tropical Forest Science, 6 (2)116-123.
11. Santamaria J and Bayman P. Fungal epiphytes and endophytes of Coffee leaves (Coffea arabica). Microbial Ecology.2005, 50 (1): 1 8.
12. Zhang H, Song Y, Tan R. Biology and Chem of endophytes. Nat. Prod. Rep.2006, 23:753-771.
13. Suryanarayanan T.S, Venkatesan and Murali T.S. Endophytic fungal communities in leaves of tropical forest trees. Diversity and Distribution patterns. Current Science. 2003, 85 (4): 489-493.
14. Ebrahimi A., Asgharian S and Habibian S. Antimicrobial activities of isolated endophytes from some Iranian native medicinal plants. Iranian J of Pharmaceutical Sciences. 2010, 6(3):217-222.
15. Tejesvi M.V, Tamhankar S.A, Kiri K.R, Rao V.S and Prakash, H.S. Fungal analysis of endophytic Pestaliopsis species from ethnopharmacologically important medicinal trees. Fungal diversity.2009, 38:167-183.
16. Radji M., Sumiati A., Rachmayani R and Elya B. Isolation of fungal endophytes from Garcinia mangostana and their antibacterial activity. African J of Biotechnology.2011, 10(1):103-107.
17. Schulz B, Wanke U, Draeger S, Aust H-J. Endophytes from herbaceous plants and schrubs: effectiveness of surface sterilization methods. Mycol. Research.1993, 97 (12):1447-1450.
18. Powthong P., Jantrapanukorn B., Thongmee A and Suntornthiticharoen

P. Screening of antimicrobial activities of the endophytic fungi isolated from sesbania grandiflora (L.) Pers. J.Agr.Sci. Tech. 2013, 15:1513-1522.

19. Jalgaonwala R, Mohite B., V. and Mahajan R. T. A review: Natural products from plant associated endophytic fungi. J.Microbiol. Biotech.Res. 2011, 1(2):21-32.
20. Ola A, Thomy D, Lai D, Brötz-Oesterhelt H and Proksch P. Inducing secondary metabolite production by the endophytic fungus Fusarium tricinctum, through co-culture with Bacillus subtilis. J. Nat. Prod.2013, 76: 2094-2099.
21. Clinical and Laboratory Standards Institute (CLSI). 2007. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard, 9th ed. CLSI document M07-A9. Wayne, Pa.
22. Arnold E and Lutzoni F. Diversity and host range of foliar fungal endophytes: Are tropical leaves biodiversity hotspots? Ecology. 2007, 88 (3): 541-549.

EVALUATION OF SAFETY AND EFFICACY OF TRIPLE DRUG FIXED DOSE COMBINATION OF VOGLIBOSE, GLIMEPIRIDE AND METFORMIN IN TYPE 2 DIABETES MELLITUS

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ABSTRACT:
Anti-diabetic therapy initiated should be directed towards the target, both fasting and as well as postprandial hyperglycaemia. Despite the introduction of new agents efforts for better management of diabetes are disappointing and the control of blood glucose levels remains unsatisfactory. The aim of the present study was to evaluate the safety and efficacy of triple drug fixed dose combination (FDC) of Voglibose, Glimepiride and Metformin in type 2 diabetes mellitus. The study was post marketing surveillance (PMS) non-randomized, open, non-comparative and multi centric study. The above mentioned FDC was administered to 118 type 2 diabetic patients once daily for 3 months. Baseline values were recorded for glycated haemoglobin (HbA1c), fasting plasma glucose (FPG) and post prandial blood glucose/hyperglycaemia (PPHG) level. There was significant decrease in HbA1c value (8.69 ± 1.81 % vs. 6.475± 0.39 %, P<0.0001); FPG (206.5±73.76 mg/dl vs. 112.7 ± 25.73mg/dl, P<0.0001) and PPHG level (244.7 ± 69.95 mg/dl vs. 141.7 ± 22.64 mg/dl, P<0.0001) after 3 month of the treatment from the baseline. The triple drug FDC of Voglibose, Glimepiride and Metformin significantly decreased the HbA1c, FPG and PPHG levels at the end of the treatment. Investigators observed good clinical effectiveness without any adverse effect reported.

Keywords: Blood Glucose, Fasting, Post-prandial, HbA1c, Triple Drug Combination
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INTRODUCTION:

Diabetes mellitus (DM) is an endocrine disorder and one of the most common non-communicable diseases globally. The prevalence of type 2 diabetes across the world has been described as a global pandemic [1]. The prevalence of diabetes is steadily increasing worldwide, particularly in the developing countries like India [2]. India had 32 million diabetics in 2000 and it is expected to increase to 80 million by 2030 [3]. The predominant clinical form of DM is Type 2 DM which accounts for more than 90 % of all the cases and is responsible for developing complications which severely alters the quality

of life and gives an enormous burden on the health care system [4]. The treatment goals in Type 2 DM are the relief of acute symptoms and prevention of long term complications, whilst avoiding hypoglycaemia. Aggressive, tight control of serum glucose reduces risk of micro vascular disease [5].

According to UKPDS 38 (The UK Prospective Diabetes Study), treating other risk factors like dyslipidemia and hypertension has been shown to be effective in reducing macro vascular disease [6].

Dietary and lifestyle modifications form the mainstay of therapy for Type 2 DM [7]. Pharmacological therapy is advocated when treatment goals are not achieved with lifestyle modifications. When the lifestyle modification, diet and exercise fails to maintain the adequate glycaemic control, oral hypoglycaemic agents are introduced as a treatment approach. Several oral anti hyperglycaemic agents are available to optimize the management of Type 2 DM [8]. Despite the introduction of new agents to the armamentarium of hypoglycaemic agents, efforts for better management of diabetes are disappointing and the control of blood glucose levels remains unsatisfactory [9].

For the optimal management of type 2 DM requires a consideration on the relationships between HbA1c, FPG and PPHG (the glucose triad), and how these changes during development and progression of the disease. Early and sustained control of glycaemia remains important in the management of type 2 DM. The contribution of PPHG levels to overall glycaemic control and the role of PPHG targets in disease management are currently debated [1]. However, many patients do not reach the HbA1c targets set according to published guidelines. The landmark UKPDS showed that every reduction of 1% HbA1c reduced the risk of all microvascular and macrovascular chronic complications [10]. Guidelines for good glycaemic control have been agreed upon and a patient is generally considered to have achieved successful disease control when their HbA1C is < 7% [11-12].

To achieve optimal glycaemic control for each patient it is likely to have to consider plasma glucose levels after the overnight fast and after meals as well as the variability of glucose levels. Hence the selection of antidiabetic therapy is important to achieve target goals of both fasting and PPHG.

Glucose triad (HbA1c, FPG and PPHG) impact in the management approaches:

By combination of lifestyle modification and appropriate drug therapy [3] it is doubtful to reach the HbA1c goal [1]. Routine measurement of PPHG levels is not currently recommended or even practical for all patients with type 2 DM. International Diabetes Federation (IDF) guidelines for the management of post meal (post-prandial) glucose states that the goal of diabetes therapy should be to achieve glycaemic status as near to normal as safely possible in all three

measures of glycaemic control, namely HbA1c, FPG and PPHG [13].

FPG and PPHG both contribute to HbA1c. Treatment of both FPG and PPHG should be initiated simultaneously at all levels of HbA1c above agreed levels. Based on hypoglycaemic mechanism of action, they are subdivided into agents that increase insulin secretion like sulfonylureas, meglitinides, GLP-1 (Glucagon-like peptide-1) agonists, DPP-4 (Dipeptidyl peptidase) inhibitor, reduce glucose production like biguanides, increase insulin sensitivity like thiazolidinediones and reduce carbohydrate absorption like α-glucosidase inhibitors. Voglibose is a competitive inhibitor of α - glucosidase enzyme present in the brush border of small intestine. It inhibits the cleavage of complex carbohydrates into simple sugars and inhibits their absorption from small intestine [14]. The primary mechanism of action of glimepiride in lowering blood glucose appears to be dependent on stimulating the release of insulin from functioning pancreatic beta cells. In addition, extrapancreatic effects may also play a role in the activity of glimepiride. Glimepiride administration can lead to increased sensitivity of peripheral tissues to insulin [15].

Metformin improves the glucose tolerance in patients with type 2 DM, lowering both the basal and postprandial plasma glucose. Metformin decreases hepatic glucose production, decreases intestinal absorption of glucose, and improves insulin sensitivity by increasing peripheral glucose uptake and utilization [16].

Current recommendations of the American Diabetes Association include a trial of diet and exercise as first line therapy for the treatment of patients with type 2 DM [17]. If glycemic control is not achieved with diet and exercise within a three-month period, pharmacologic intervention is required. Moreover if adequate control is not obtained with the use of a single agent, combination therapy is an option. Several of the available oral agents have been studied in combination and have been shown to further improve glycemic control when compared to monotherapy [18]. Some physicians now advocate the therapy combining three oral agents (sulfonylurea, metformin, alpha-glucosidase inhibitor or sulfonylurea, metformin, thiazolidinedione) in the management of type 2 DM [19]. This study was conducted to find the efficacy of triple drug combination (i.e. Voglibose, Metformin and Glimepiride) in the management of type 2 DM based on its effect on all the three parameters of glucose triad FPG, PPHG and HbA1c levels.

MATERIALS AND METHODS:

This study was a non-randomized, open, non-comparative, multi centric and post marketing surveillance study. The fixed dose combination (FDC) of Voglibose, Glimepiride and Metformin was orally administered as once daily to type 2 diabetic patients for 3 months. An approval from ethics committee was not obtained since

this was a post marketing surveillance study and the investigational product is already in market and approved by regulatory authorities. Informed consent was obtained from the patients and the post marketing surveillance was done in accordance with the clinical principles laid down in declaration of Helsinki [20]. A total of 118 type 2 diabetic patients were enrolled from the diabetic clinic and completed the treatment. At the time of entry into the study, base-line demographics were recorded. Patients were observed on 1st month of treatment, then subsequently 2nd and 3rd month of the treatment. Evaluation of FPG and PPHG were carried out regularly at the interval of each month for all the enrolled patients. The HbA1c level was examined only before the treatment and after 3 months of treatment. The HbA1c determination was carried out by using BIORAD Micromat II HbA1c instrument [21], while FPG and PPHG were determined by using Microplate reader [22].

Inclusion Criteria:

Both male and female patients over 32 years of age, HbA1c >7%, FPG level >200 mg/dl and PPHG >240 mg/dl were included in the study. Clinical criteria for the evaluation included FPG, PPHG and HbA1c value. After informed consent was obtained, patients were prescribed to receive the FDC of Voglibose, Glimepiride and Metformin tablet once daily for three months.

Exclusion Criteria:

Patients with current insulin therapy or received insulin for more than six weeks in last 3 months, who had known hypersensitivity to biguanides and sulphonylurea, who were on chronic medication known to affect glucose metabolism were excluded from the study. In addition, patients with renal disease or renal dysfunction, with congestive heart failure, hepatic insufficiency, alcoholics, pregnant and lactating women were excluded from the study.

Efficacy and Safety Evaluations:

The primary efficacy variable was the change in HbA1c from baseline to 3 month. Secondary efficacy outcomes included changes in FPG and 2-hour PPHG levels from baseline to the subsequent month of the treatment up to 3 month. Safety outcomes included adverse events, particularly hypoglycaemic symptoms. The patients were interviewed and asked for any type of adverse events throughout the study. The patients were specially asked for the hypoglycaemic symptoms during each visit to the study centre. The daytime hypoglycaemic episodes are usually recognized by sweating, nervousness, tremor, and hunger while night-time hypoglycaemia may be without symptoms or manifest as night sweats, unpleasant dreams, or early morning headache [23].

Statistical Analysis:

The analysis of HbA1c and FPG and PPHG was carried out by using graph pad prism 6.

Comparison between the baseline values with the value on the 1st, 2nd and 3rd month of treatment were made, as well as comparison in between these months was done by applying one way analysis of variance and the Turkeys multiple comparison test. Values of P <0.001 were considered significant.

RESULTS:

A total of 118 patients were screened and completed the study. The baseline characteristics of all patients at randomization are summarized in table 1.

Table 1: Baseline characteristics of all patients

Number of patients	118 (54% Male; 46% Female)
Age (yrs)	32 - 85
HbA1c (%)	8.69 ± 1.81
FPG (mg/dl)	206.5±73.76
PPHG (mg/dl)	244.7 ± 69.95
Body weight (kg)	69.91±12.55

Evaluation of Glycaemic Control:

Glycated haemoglobin (HbA1c):

HbA1c level was significantly reduced from the baseline after using the triple combination of voglibose, glimepiride and metformin. During the study there was significant differences found in the value of HbA1c at the baseline to the value observed after the completion of the treatment, respectively (8.69 ± 1.81 vs.6.475± 0.39, P< .0001) as shown in table 2.

Table 2: FPG, PPHG, and HbA1c values from the baseline and after 30, 60 and 90 days (Mean±SD)

Parameters	Baseline	Day 30	Day 60	Day 90
FPG (mg/dl)	206.50±73.76	155.70±46.77***	133.30±29.29***#	112.70±25.73***
PPHG(mg/dl)	244.70±69.95	193.50±44.5***	169.60±40.0***#	141.70±22.64***
HbA1c (%)	8.69±1.81	8.06±1.35***	7.07±0.89***	6.47±0.39***$

*** P< 0.0001 vs. Baseline, # P< 0.01 vs Day 30, $ P< 0.01 vs. Day 60

Evaluation of Fasting Plasma Glucose (FPG) level:

The FPG level was reduced throughout the study period of 3 month. The FPG level was measured at baseline and then subsequently at 1st, 2nd and 3rd month of the treatment. The FPG level was 206.5±73.76 mg/dl at baseline. The FPG level was significantly reduced just after 1 month of the treatment from the baseline value (206.5±73.76 mg/dl vs. 155.7 ± 46.77 mg/dl, p=0.01). The level of significance was highest between the FPG at baseline and on 3rd months of the treatment (206.5±73.76 mg/dl vs. 112.7 ± 25.73 mg/dl, P< .0001). There was no significant change between the 1st month and 2nd month of the treatment (155.7 ± 46.77 mg/dl vs. 133.3 ± 29.29 mg/dl, P=0.01) and between 2nd and 3rd month of the treatment (133.3 ± 29.29 vs. 112.7 ± 25.73, P=0.01). Overall the FPG level was significantly (P<0 .0001) decreased by 93.8± 48.03 mg/dl from the baseline after the completion of the study of 90 days (Table 3). The decrease in FPG level from the baseline to the subsequent month of treatment is presented in table 3.

Table 3: Reduction in FPG, PPHG and HbA1c from the baseline (Mean±SD)

Parameters	Day 30	Day 60	Day 90
ΔFPG (mg/dl)	-50.8±26.99***	-73.2±44.47***	-93.8±48.03***
ΔPPHG(mg/dl)	-51.2±25.45***	-75.1±29.93***	103.0±47.31***
ΔHbA1c (%)	-0.62±0.46***	-1.62±092***	-2.22±1.42***

*** P<0 .0001 vs. Baseline

Evaluation of Post Prandial Blood Glucose (PPHG) level:

The PPHG level was reduced throughout the study period of 3 month. The PPHG level was measured at baseline and then subsequently at 1st, 2nd and 3rd month of the treatment. The PPHG level was 244.7 ± 69.95 mg/dl at baseline. The PPHG level was significantly reduced on 2nd month of the treatment vs. baseline (244.7 ± 69.95 mg/dl vs. 169.6 ± 40.02 mg/dl, P=0.01). But the level of significance was highest between the PPHG at baseline and to that on the 3rd month of the treatment (244.7 ± 69.95 mg/dl vs.141.7 ± 22.64, P< 0.0001). By applying the turkey's multiple comparison it was observed that there was no significant changes in PPHG level between the1st month of treatment to the 2nd month of treatment (193.5± 44.5 mg/dl vs. 169.6 ± 40.02mg/dl, P=0.01) and also in

between 2nd and 3rd month of the treatment (169.6 ± 40 mg/dl vs. 141.7 ± 22.64 mg/dl, P= 0.01). Overall the PPHG level was significantly decreased by 103 ± 47.31 mg/dl from the baseline after the completion of the study period of 90 days (Table 3).

Evaluation of body weight variation during the study period:

There were no significant changes observed in body weight during the whole treatment period. Body weight was recorded as mean average (±SD) at the entry of the study, and then subsequently on 1st , 2nd and on 3rd month of the treatment (69.91±12.55 vs70±12.53, 69.7± 12.27 and 69.25± 12.57 kg respectively).

Evaluation of Hypoglycaemic and other adverse effect:

The patients were interviewed during each visit and at the end of the study for the detection of any hypoglycaemic episode and about other side effects like nausea, vomiting, headache. No patient complained about the side effects including nausea, vomiting, headache or GIT side effects at the given doses of medication.

Evaluation of Global efficacy and tolerability:

As per investigators assessment about efficacy and tolerability of Voglibose, Metformin and Glimepiride tablet, all (100%) the patient tolerated the treatment and benefitted. Moreover investigator showed the interest that it would be the good choice in the management of diabetes when there is need to control both FPG and PPHG level.

DISCUSSION:

The management of DM includes diet control, exercise and pharmacological therapy. PPHG, similar to post-challenge glucose, was related to cardiovascular disorders (CVD) than FPG [24-26]. However, higher fasting hyperglycaemia was not significantly associated with CVD risk. Previous analyses suggested that fasting hyperglycaemia tended to be associated with beta cell dysfunction, whereas post-challenge hyperglycaemia tended to be more strongly related to insulin resistance, higher blood pressure, obesity, and dyslipidemia [27]. The drug therapy is generally initiated either with sulfonylurea or metformin as monotherapy. In the present study patients with DM whose glycaemic status were not controlled with two oral hypoglycaemic agents (metformin and glimepiride) were given third drug voglibose as FDC. The effect of add on therapy with voglibose as a third agent was observed on various parameters. Among the clinical parameters, there was no significant change observed in body weight at the end of study. A significant reduction in FPG, PPHG and HbA1c was found with FDC.

For the optimal management of type 2 DM there is the requirement to understand the relationships between HbA1c, FPG and PPHG level (the glucose triad), and how these change

takes place during development and progression of the disease. Early and sustained control of glycaemia remains important in the management of type 2 DM. When antidiabetic therapy is initiated, physicians may need to consider selection of agents that target both fasting and PPHG levels. M. John et al [28], in a review article reported that, triple FDCs provide effective glycemic control in a safe, well tolerated, and economic manner. They also stated that, the components of FDC acts by different mechanisms thereby targeting multiple pathophysiological targets. Similar results were also reported by C Rao et al [29], concluding their findings as triple drug combination of voglibose, metformin and glimepiride reduces HbA1c, FPG and PPHG level in type 2 DM patients. In the same study, they reported that, the above mentioned triple drug FDC was safe and well tolerated in their clinical trial. Jindal et al [30] in another trial studied the effect of addition of voglibose to the combination of glimepiride and metformin and observed changes on various parameters i.e. FPG, PPHG, HbA1c and lipid profile (Total cholesterol, triglycerides, low density cholesterol, and very low density cholesterol and high density lipoprotein) in type 2 DM patients. At the end of the study it was found that there was a significant reduction in FPG, PPHG and HbA1C was found with the addition of voglibose. The reduction in these parameters was observed in chronological sequence with duration of study i.e. at 1st, 2nd, 3rd, 4th, 5th and 6th months. Addition of voglibose was reported to have an influence on serum lipids, which include total cholesterol, triglycerides, low density cholesterol and very low density cholesterol; these were reduced significantly with voglibose [30]. Derosa et al [31] also observed significant reduction in FPG, PPHG and HbA1c with combination of sulfonylurea, metformin and acarbose.

In this post marketing surveillance study, HbA1c value was significantly reduced from the baseline after using the FDC of voglibose, glimepiride and metformin. During the study there was significant differences found in the value of HbA1c at the baseline to the value observed after the completion of the treatment (8.69 ± 1.81 to 6.475± 0.39, P< 0.0001).

The FPG level was reduced throughout the study period of 3 months. FPG level was significantly (P< 0.0001) decreased by 93.8 ± 48.03 mg/dl from the baseline after the completion of the study of 3 months. After 3 month the FPG level was reduced to 112.7 ± 25.73 mg/dl. Moreover the PPHG level was also reduced throughout the study period of 3 month. Overall the PPHG level was decreased significantly by 103 ± 47.31 mg/dl from the baseline after 3 months of treatment and the PPHG level lowered down to the value 141.7 ± 22.64 mg/dl.

Overall this combination is highly effective and safe in controlling all the glycemic parameters like HbA1c, FPG and PPHG for optimal management of type 2 DM. Regarding the

tolerability and safety of the FDC of Voglibose, Metformin and Glimepiride, 100% of patients tolerated the treatment very well without the need for discontinuing the therapy due to adverse effect.

CONCLUSION:

The FDC of Voglibose, Metformin and Glimepiride significantly decreased the HbA1c value, FPG and PPHG level after 3 months of treatment from the baseline. Investigators observed that the therapy was safe and well tolerated. Investigators commented that this is a good option to control the FPG and PPHG level for the optimal management of type 2 DM.

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Competing Interests:

All authors had access to the data and vouch for the veracity and completeness of the data and the data analysis. Authors have declared that no competing interests exist.

REFERENCES:

1. Ceriello A. The glucose triad and its role in comprehensive glycaemic control: current status, future management: Int J Clin Pract, November 2010; 64(12): 1705–1711
2. King H, Aubert RE, Herman WH. Global burden of diabetes, 1995-2025: prevalence, numerical estimates and projections. Diabetes Care.1998; 21: 1414-1431.
3. Ramchandran A, Snehlata C, Vijay V. Burden of type 2 diabetes and its complications – The Indian scenario. Current Sciences. 2002; 83: 1471-1476.
4. Nathan DM, Meigs J, Singer DE. The epidemiology of cardiovascular disease in type 2 diabetes mellitus: how sweet it is...or is it? Lancet. 1997; 350 (1): 4-9.
5. Hu FB, Manson JE, Stampfer MJ.Colditz G, Liu S, Solomon CG, Willett WC. Diet, Lifestyle and the risk of type 2 diabetes mellitus in women. N Engl J Med. 2001; 345: 790-797.
6. Guillausseau PJ, Massin P, Charles MA. Allaguy H, Güvenli Z, Virally M, Tielmans D, Assayag M, Warnet A, Lubetzki J. Glycaemic control and development of retinopathy in type 2 diabetes mellitus: a longitudinal study. Diabetes Med. 1998; 15: 151-155.
7. Grundy SM. Dietary therapy in diabetes mellitus. Diabetes Care. 1991; 14: 796-808.
8. Giuseppe Derosa and Salvadeo Sibilla Optimizing combination treatment in the management of type 2 diabetes Vasc Health Risk Manag. 2007Oct;3(5):665–671
9. Nathan DM, Kitrick C, Larkin M, Schaffran R, Singer DE. Glycemic control in diabetes mellitus: have changes in therapy made a difference? Am J Med 1996; 100:157-63.
10. UK Prospective Diabetes Study Group. UK Prospective Diabetes Study 16 (Overview of 6 years' therapy of type II diabetes: a progressive disease). Diabetes 1995; 44:1249–1258.
11. American Diabetes Association. Standards of medical care in diabetes Diabetes Care 2008; 31(Suppl. 1): S12–54.
12. Canadian Diabetes Association. Canadian Diabetes Association 2008 clinical practice guidelines for the prevention and management of diabetes in Canada Can J Diabetes 2008; 32(Suppl. 1): S1–201.
13. Ceriello A, Colagiuri S, Gerich J, Tuomilehto J. Guideline for Management of Postmeal Glucose. Brussels: International Diabetes Federation, 2007.

14. Powers AC. Diabetes Mellitus. In: Fauci AS, Braunwald E, Kasper DL, Stephen LH, Dan LL, Jameson LL, Loscalzo J. Harrison's principles of internal medicine. 17th ed. New York: McGraw Hill 2008; 2275- 2304.
15. AMARYL tablets (Glimepiride) Prescribing Information, Sanofi-Aventis U.S. LLC, Revised May 2009
16. Glucophage (metformin hydrochloride) Tablets Prescribing Information, Bristol-Myers Squibb, NJ 08543 USA
17. American Diabetes Association. The pharmacological treatment of hyperglycemia in NIDDM. Diabetes Care 1995; 18:1510-8.
18. Riddle M. Combining sulfonylureas and other oral agents. Am J Med 2000; 108 (suppl 6a):15S-22S.
19. Ovalle F, Bell DSH. Triple oral antidiabetic therapy in type 2 diabetes mellitus. Endocr Pract 1998;4:146-7
20. World Medical Association Declaration Of Helsinki Ethical Principles for Medical Research Involving HumanSubjects: www.wma.net/en/30publications/10policies /b3/17c.pdf assessed on July 2015
21. Bio-Rad Laboratories Hemoglobin Testing: www.bio-rad.com/webroot/web/pdf/cdg/literature/A196_dg101143_diabetes.pdf assessed on July 2015
22. Bio-Rad Life sciences research: http://www.bio-rad.com/en-in/category/microplate-readers assessed on July 2015
23. R. D. Shimpi, P. H. Patil, V. G. Kuchake, P.V.Ingle, S. J. Surana, P. N. Dighore; Comparison of effect of metformin in combination with glimepiride and glibenclamide on glycaemic control in patient with type 2 diabetes mellitus International J of PharmTech Research Jan – March 2009 Vol.1,No.1,pp 50-61
24. Glucose tolerance and mortality: comparison of WHO and American Diabetes Association diagnostic criteria. The DECODE study group. European Diabetes Epidemiology Group. Diabetes Epidemiology: Collaborative analysis Of Diagnostic criteria in EuropeLancet 1999; 354: 617–621.
25. Glucose tolerance and cardiovascular mortality: comparison of fasting and 2-hour diagnostic criteria, Arch. Intern. Med. 2001; 161 397–405.
26. F. Cavalot, A. Petrelli, M. Traversa, K. Bonomo, E. Fiora, M. Conti, Anfossi G, Costa G, Trovati M. Postprandial blood glucose is a stronger predictor of cardiovascular events than fasting blood glucose in type 2 diabetes mellitus, particularly in women: lessons from the San Luigi Gonzaga Diabetes Study, J. Clin. Endocrinol. Metab. 91 (2006) 813–819.
27. Kuo-LiongChiena,b, Bai-Chin Lee b, Hung-Ju Lin b, Hsiu-Ching Hsu b, Ming-Fong Chen; Association of fasting and post-prandial hyperglycemia on the risk of cardiovascular and all-cause death among non-diabetic Chinese: Diabetes research and clinical practice 2009; 83:e4 7– e50
28. MathewJohn, DeepaGopinath, and Sanjay Kalra Indian J Endocrinol Metab. 2015 May-Jun; 19(3): 311–313. Triple fixed drug combinations in type 2 diabetes
29. C Rao, Dr. A. A. Faruqui Efficacy and safety of oral triple drug combination (voglibose, glimepiride and metformin) in the management of type 2 diabetes mellitus Int J Cur Res Rev, Aug 2013/ Vol 05 (16) 20-26
30. Dr. Amita Jindal, Dr. Mahesh Jindal, Dr. Mandeep Kaur, Mr. Raj Kumar, Mr. Rajender Singh Brar. Efficacy and safety of voglibose as an add-on triple drug in patients of type two diabetes mellitus uncontrolled with glimepiride and metformin in Punjabi population Indian Journal of Basic and Applied Medical Research; June 2014: Vol.-3, Issue- 3, P. 111-116
31. Derosa G, Salvedeo S, Angelo A, Ferrari I, Moreu R, Palumbo I et al. Metabolic effect of repaglinide or acarbose when added to a double oral antidiabetic treatment with sulphonylureas and metformin: a double blind, cross over, clinical trial. Informa Healthcare. 2009; 25 (3): 607-615.

EFFECT OF DIFFERENT DEGREES OF TILT ON HEART RATE, PULSE PRESSURE AND MEAN ARTERIAL BLOOD PRESSURE IN YOUNG MALE AND FEMALE NIGERIANS

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ABSTRACT:

It is well documented that changes in cardiac output are lower in the upright than supine positions. This study investigates the effect of different angles of tilt on the heart rate, pulse pressure and mean arterial pressure (MAP) of healthy male and female subjects in the 18 to 24 years age group and heights between 150 -180cm. All subjects were students in the University of Ilorin. Each participant was studied and the parameters were determined at the supine position and head up tilts at 15º, 30º, 45º and, 60º for 15 minutes interval. Additionally the supine position variables were taken as the resting value against each of the head up tilt angle degree. The result shows that there was progressive and significant increase in heart rate from supine 59.5±1.3beat/min to 62.2 ± 1.4beat/min ($p<0.05$) at 15º; 59.5±1.3beat/min to 63.8 ± 1.4beat/min at 30º tilt ($p<0.05$); 59.5 ± 1.3beat/min to 65.9 ± 1.4beat/min at 45º tilt ($p<0.05$); 59.5 ± 1.3beat/min and 68.2 ± 1.6beat/min at 60º tilt ($p<0.05$). The results also showed that there were significant ($p<0.05$) increases in pulse pressure at all angles which peaked at 30º tilt. Furthermore, it was observed that MAP significantly ($p<0.05$) increased with corresponding increase in the angle of tilt from supine to 60º (76.5±2.2 mmHg (supine) to 80.6±2.1 mmHg (15 º) to 83.8±2.1 mmHg (30 º) to 85.4±2.1 mmHg (45 º) to 85.4±2.2 mmHg (60 º). Our results demonstrated that the heart rate, pulse pressure and MAP were significantly ($p<0.05$) increased in males and females for all the variables.

Keywords: Tilt, heart rate; pulse pressure; mean arterial blood pressure
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INTRODUCTION:

Many workers have shown that the cardiac output in a healthy individual is less in the upright than in the recumbent position [1, 2]. During a moderate posture change in human from sitting to supine, mean arterial blood pressure (MAP) decreases [1, 2]. The mechanisms for this involve cardiopulmonary low and arterial high pressure receptor stimulation which induces peripheral vasodilatation and decrease in heart rate [3]. The muscle vascular bed is sensitive to changes in baro-receptor stimulation [4]. Many senescent individuals demonstrate an ability to regulate MAP in response to standing or head-up tilt [5]. In some individuals, sudden standing causes a fall in blood pressure, dizziness, dimness of vision and fainting. The causes of this orthostatic (postural) hypotension are multiple. The major compensation on assuming the upright positions are triggered by drop in blood pressure in the carotid sinus and aortic arch [3, 6, 7, 8]. The heart rate increases helping to maintain cardiac output. There is relatively little vasoconstriction in the periphery and arterial pressure as head level drops but jugular venous pressure falls reducing the drop in perfusion pressure (arterial pressure- venous pressure) [9].

Generally, increases in cardiac output increases the systolic pressure whereas increase peripheral resistance increases the diastolic pressure. The physiological events underlying all these aforementioned changes are complex and depend on many variables, such as the angle of tilt and length of time the patient remains at a given angle and whether the change is from recumbence to upright position or vise-versa. Posture and its relation to cardiac output is important with the introduction of hypotensive drugs [10]. This study aimed to determine the relationship between angle tilt and changes in heart rate, pulse pressure and mean arterial blood pressure in young adult Nigerians.

SUBJECTS AND METHODS:

Fifty healthy volunteers (30 males and 20 females) who were students in the University of Ilorin participated in this study. The age range was between 18 and 24 years, heights between 150 to 180cm and weights between 55 to 77kg. The volunteers were normotensive (supine blood pressures; not greater than 120/80 mmHg, were not taking medications, and had no known cardiovascular diseases. The volunteers were requested to reframe from drinking caffeine and alcohol, and not to be involved in intensive exercise 24 hours before the study if they were not accustomed to physical exercise.

This study followed the guidelines approved by the Ethical committee of the University of Ilorin,

and a written informed consent was obtained from each volunteer.

The heart rate was measured using standard (automated) digital sphygmomanometer and was confirmed manually using radial artery. The systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured using the automated sphygmomanometer and the blood pressure readings confirmed by aneroid manual sphygmomanometer. The pulse pressure (PP) was estimated by the difference between systolic and diastolic blood pressure that is calculated using formula: PP= SBP-DBP. The mean arterial blood pressure was determined indirectly by DBP plus one third of pulse pressure (MAP=DBP+1/3PP) [10].

Studies were conducted three hours after breakfast or lunch [10]. The subject was weighed and height determined. Thereafter, the subject was placed on the tilt bed, maintained in a supine position and heart rate and arterial blood pressure were determined. All subjects were maintained in the supine position for thirty (30) minutes before readings were taken. The variables measured were; heart rate, systolic and diastolic pressures. The experiment was done for four (4) different degrees of tilt [10].

The tilt positions were 15º, 30º, 45º and 60º. Each subject was tilted to all these positions sequentially one after the other starting from the least angle; and readings were taken at 15 minutes interval for each angle. The passive postural changes were performed by manual tilt bed. The duration of the procedure was about two hours for each subject [10].

Percentage change was calculated as follows [10]:

$$\text{Percentage change } (\%\,\Delta) = \frac{(b-a)\ x\ 100}{a}$$

Where a= values of variables at rest (supine position) and
b= values of variables after each tilts

The mean values of heart rate, pulse pressure and MAP were determined and the standard errors of the means were calculated. Significant responses were evaluated by Student t-test for paired comparison [11]; $P \leq 0.05$ was taken as statistically significant. The Student paired t-test was used to detect whether means differed at similar points in time, comparing values of corresponding series (supine vs 15º tilt, supine

vs 30º tilt, supine vs 45º tilt and supine vs 60º tilt).

RESULTS:

Effect of different degrees of tilt on the male subjects:

All the male subjects showed significant increase in heart rate at 15º tilt after 15 minutes interval of postural change (supine to 15º tilt). The mean value increase from 59.5±1.3beat/min to 62.2 ± 1.4beat/min (an increase of 4.7 ± 1.1%, $p<0.05$, n=30). Continuous increase in heart rate was also observed as the tilt angles increases, after 15 minutes interval. The overall mean heart rate increases with changes in tilt angle, supine and 30º tilt: 59.5 ± 1.3 and 63.8 ± 1.4beat/min; supine and 45º tilt: 59.5 ± 1.3 and 65.9 ± 1.4beat/min; supine and 60º tilt: 59.5 ± 1.3 and 68.2 ± 1.6beat/min. The change in heart rate was significant as shown in the table 1.

All the male subjects also showed increase in pulse pressure at various angles of tilt and the increase was highest at angle 30º tilt.

At angle 45º and 60º tilt, the pulse pressure fell from the value obtained in 30º tilt. Hence, mean pulse pressure significantly increased from supine position to 30º tilt as shown in table 1.

As can be seen in Table 1, all the male subjects showed increase in MAP and the increase was in line with increase in the angle of tilt. Overall, MAP increases with change in angle of tilt. The changes were significant ($p<0.05$).

Table 1: Effect of different degrees of tilt on heart rate, pulse pressure and mean arterial blood pressure for the male and female subjects

Subjects		Supine (a)	15º tilt (b)	30º tilt (b)	45º tilt (b)	60º tilt (b)
Male (n=30)	**Heart Rate (bpm)**	59.5±1.3	62.2±1.4*	63.8±1.4*	65.9±1.4*	68.2±1.6*
	Pulse Pressure (mmHg)	39.0±1.1	42.4±1.2*	46.7±1.1*	44.1±1.0*	41.6±0.8*
	MAP (mmHg)	76.5±2.2	80.6±2.1*	83.8±2.1*	85.4±2.1*	85.4±2.2*
Female (n=20)	**Heart Rate (bpm)**	66.1±1.7	68.4±1.8*	69.7±1.8*	71.4±1.8*	74.3±1.7*
	Pulse Pressure (mmHg)	39.4±0.9	42.0±0.9*	44.7±0.9*	41.9±1.0*	41.0±0.9*
	MAP (mmHg)	78.4±2.1	82.7±1.8*	85.2±1.8*	85.4±2.1*	86.4±1.9*

Data are Mean ± SEM values, *P<0.05 significantly different from control: supine vs 15 º tilt, supine vs 30 º tilt, supine vs 45º tilt, supine vs 60º tilt. Where a= values of variables at rest (supine position) and b= values of variables after each tilts

Table 2: Percentage changes of heart rate, pulse pressure and mean arterial blood pressure for the different degrees of tilts compared to the supine for the male and female subjects

Subjects		15º tilt (a)	30º tilt (a)	45º tilt (a)	60º tilt (a)
Male (n=30)	Heart Rate (bpm)	4.7±1.1*	7.2±1.4*	10.8±0.7*	14.7±1.3*
	Pulse Pressure (mmHg)	8.9±1.6*	21.1±3.4*	14.4±2.8*	8.0±2.4*
	MAP (mmHg)	5.8±1.1*	10.0±0.9*	12.3±1.2*	12.1±1.3*
Female (n=20)	Heart Rate (bpm)	3.5±0.5*	5.4±0.6*	8.0±0.7*	12.6±0.8*
	Pulse Pressure (mmHg)	6.8±1.1*	13.7±1.8*	6.5±1.7*	4.2±1.4*
	MAP (mmHg)	5.8±1.2*	9.1±1.3*	11.8±1.5*	10.7±1.7*
	*p-value	<0.05	<0.05	<0.05	<0.05

Data are Mean ± SEM values, a = values of variables after each tilts.

Effect of different degrees of tilt on the female subjects:

All the female subjects showed significant increase in heart rate as the angle of tilt increases. The mean heart rate increased from supine to 15º tilt, 30º tilt, 45º tilt and 60ºtilt; the corresponding values were 66.1 ± 1.7 and 68.4 ± 1.8beat/min, 66.1 ± 1.7 and 69.7 ± 1.8beat/min, 66.1 ± 1.7 and 71.4 ± 1.8beat/min, 66.1 ± 1.7 and 74.3 ± 1.7beat/min. The changes in heart rate were significant ($p<0.05$) as shown in Table 1. Increase in pulse pressure at various angles of tilt was observed for all the female subjects; the increase was highest at angle 30º tilt (Table 1). At angle 45º tilt and 60º tilt, the pulse pressure fell from the value obtained in 30º tilt. The mean pulse pressure significantly increased ($p<0.05$) from supine position to 30º tilt as shown in tables 1 and 2. The MAP for the female subjects increased from supine to the different angles of tilts. The changes in the angles of tilts are presented in tables 1 and 2.

DISCUSSION:

The subjects were selected based on their health status. Subjects with history of cardiovascular diseases, hypertension, smokers, and history of kidney diseases were excluded from the study. This is because the major products of tobacco combustion, nicotine and carbon dioxide are potent vasoconstrictors [12, 13]. Besides, nicotine stimulation increased secretion of catecholamine. The net effect is that there will be increase in peripheral

vascular resistance and this can lead to elevation of blood pressure [13].

The results obtained for both male and female subjects showed that slight increase in heart rate occurred during the first 15 minutes of tilting to the head-up position of 15^0 and thereafter increase progressively as the angle of head up position was increased to 30^0 tilt, 45^0 tilt and 60^0 tilt as presented in table 2. When compared with the recumbent values, the average heart rate at those angles was increased. The changes were significant at $p< 0.05$. The increase in heart rate which began at 15^0 tilt and gradually increased after, and the probable simultaneous augmentation of myocardial contractility (also caused by a reflex increase of sympathetic nervous system activity) prevented further decrease in cardiac output [14]. However, the increase in heart rate is in agreement with the findings of Tuckman and Shillingford [10]. The increase might be due, in part, to withdrawal of vagal inhibition [15]. In addition, in female, the sort of heart rate maintenance could be linked with estrogen. Estrogen is said to be involved in cardio-protection in female [16, 17].

As shown in table 1, there was significant increase in pulse pressure when comparing recumbence with changes in various angles of tilts. The pulse pressure increase gradually at angle 15^0 tilt (8.0±1.1%) and peak at 30^0 tilt (18.0±2.2%) before decline at angle 45^0 tilt and 60^0 tilt (11.0±1.9% and 6.5±1.6%). Generally, the average pulse pressure increased at all positions of tilt when compared to supine. The changes were statistically significant ($p<0.05$). It is well established that a change from recumbence to the vertical is accompanied by an increase in heart rate and diastolic pressure with little or no increase in systolic pressure [18]. The fall in pulse pressure at angles 45^0 and 60^0 is in agreement with findings of Tuchman and Shillingford that “It may well be that the reflexes associated with control of diastolic pressure are more active at lesser degrees of tilt than those responsible for the increase in cardiac rate” [10]. Increase in systolic blood pressure could be due in part to increase in alpha adrenergic activity [9, 15].

There was progressive increase in MAP as the angle of tilt increases. The MAP increased gradually at 150 and markedly at other angles of tilt. When comparing supine to these angles, however, the changes were significant (Table 2). When the vascular bed of the legs are separated from circulation by arterial occlusion, the decrease in MAP during posture change from upright, sitting to supine vary similar to those during posture change without occlusion [19]. This is in central effect of water immersion, where vasodilation in the legs is necessary to prevent MAP from increasing [19]. It has been observed that during posture change from sitting to supine, with a

subsequent stimulation of arterial high and cardiopulmonary low pressure receptors, MAP decreases [20]. During orthostasis which is the maintenance of an upright standing posture, the splanchnic regions accounts for some 30% of the increase in total peripheral resistance, while skin and muscles account for some 40% of the increase in total peripheral resistance [3]. It was expected that constriction in both the vascular beds would be pivotal to inducing an increase in MAP during a moderate anti orthostatic posture change.

CONCLUSION:

The effects of different angles of head-up tilting on heart rate, pulse pressure and mean arterial blood pressure have been studied on male and female subjects in the 18 to 24 years age group. The results show that at head up tilt position of 15^0, 30^0, 45^0 or 60^0 major change of pulse pressure occurs at 30^0 and there is little change on further tilting; heart rate increase with increase in angle of tilt.

The major change of mean arterial pressure occurs at 30^0 and 45^0.

REFERENCES:

1. McMicheal J, Sharpey-Schafer EP. Cardiac output in man by direct Fick method: effects of posture, venous pressure changes atropine and adrenaline. British Heart Journal 1944; 6: 33.
2. Reeves JJ, Grover RF, Blount SG, Filley FG. Cardiac output response to standing and treadmill walking. J.appl.Physiol. 1961; 16: 283.
3. Rowell LN. Human Cardiovascular control. Oxford University Press.1993
4. Öberg B. Effects of cardiovascular reflexes on net capillary fluid transfer. Acta Physiologica Scandinavica. 1964;62(suppl. 229):1–98
5. Burton AC. Physiology and biophysics of the circulation. 2nd Ed. Chicago, Year Book Medical Publishers. 1972.
6. Eckberg DL, Sleight P. Human baroreflexes in health and disease. Monograph of the Physiological Society . Oxford:Clarendon Press, 1992.
7. Wieling W, Karemaker JM. Measurement of heart rate and blood pressure. In: Mathias C, Bannister R, eds. Autonomic Failure. Oxford, UK: Oxford University Press, 2013; in press.
8. Cooper VL, Hainsworth R. Carotid baroreceptor reflexes in humans during orthostatic stress. Exp Physiol 2001;86:677–681
9. Ramsey, M W. Effects of head-up tilt on mean arterial pressure, heart rate and regional cardiac output distribution in aging rats. Doctoral dissertation, Texas A & M University, 2005
10. Tuckman J, Shillingford J. Effect of different degrees of tilt on cardiac output, heart rate and blood pressure in normal man. Brit. Heart J. 1966; 28: 32-39.
11. Kennedy JB, Neville AM. Basic statistical methods for Engineers and Scientists (2nd ed). Harper and Row, New York.1976; pp. 205 - 212.
12. Gerriten J, Ten Voorde BJ, Dekker JM, Kostense PJ, Bouter LM, Heethaar RM. Baroreflex sensitivity in the elder: influence of age, breathing and spectral methods. Clin,sci (Lond). 2000; 99: 371-381.
13. Binanay C, Califf RM, Hasselblad V, O'Connor CM, Shah MR, Sopko G, Stevenson LW, Francis GS, Leier CV, Miller LW; ESCAPE Investigators and ESCAPE Study Coordinators. Evaluation study of congestive heart failure and pulmonary artery catheterization effectiveness: the ESCAPE trial.JAMA. 2005;294(13):1625-1633.
14. Sarnoff SJ, Gilmore JP, Brockman SK, Mitchell JH, Linden RJ. Regulation of ventricular contraction by the carotid sinus: its effect on atrial and ventricular dynamics Circulat Res. 1960; 8: 1123-1136.

15. Freyschuss U. Cardiovascular adjustment to somatomotor activation. The elicitation of increments in heart rate, aortic pressure and venomotor tone with the initiation of muscle contraction. Acta Physiol Scand Suppl. 1970; 342:1-63.
16. Mendelsohn ME, Karas RH. The protective effects of estrogen on the cardiovascular system. N Engl Journal Med. 1999; 340: 1801–1811.
17. Weissman A, Lowenstein L, Tal J, Ohel G, Calderon I, Lightman A. Modulation of heart rate variability by estrogen in young women undergoing induction of ovulation. Eur J Appl Physiol. 2009;105: 381–386.
18. Hellebrant FA, Franseen EB. Physiological study of the ventrical stance of man. Physiol Rev. 1943; 23: 220.
19. Johansen LB, Jensen TVS, Pump B, Norsk K. Contribution of abdomen and legs to central blood volume expansion in human during immersion. J. Appl Physiol. 1997; 83: 695-699.
20. Pump B, Christensen NJ, Videback R, Werberg J, Hendryksen O, Norst P, Left atrial distention and antiorthostatic decrease in arterial pressure and heart rate in humans. Amer. J. Physiol, 1997; 83: 695-699.
21. Hughson RL, Edwards MR, O'Leary DD, Shoemaker JK.. Critical analysis of cerebrovascular autoregulation during reported head up tilt: 2001; 32:2403-2408.
22. Zaidi A, Benitez D, Gaydecki P, Vohra A, Fitzpatrick A. Haemodynamic effects of increasing angle of head up tilt. Heart. 2000; 83(2): 181–184.

CASE REPORT

VARIATION IN THE TERMINATION OF MUSCULOCUTANEOUS NERVE

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ABSTRACT:
A variation of the musculocutaneous nerve was observed during routine dissection for first year medical students, in a 67 year old embalmed male cadaver. The termination of musculocutaneous nerve in left upper limb was variable. After piercing the coracobrachialis muscle, musculocutaneous nerve divided into lateral cutaneous nerve of the forearm and gave an accessory branch that joined with median nerve, lateral to the insertion of the coracobrachialis muscle. Knowledge of these variations is of great importance to surgeons and orthopaedicians who do surgical interventions in that region.

Key Words: musculocutaneous nerve, coracobrachialis muscle, median nerve, variations
Submitted April 2015, Accepted August 2015

INTRODUCTION:

Brachial plexus is formed by the anterior primary rami of spinal nerves C5, C6, C7, C8 and T1 [1]. The fibers of the plexus may be joined by branches from the fourth cervical and second thoracic nerves forming a prefixed or postfixed plexus. C5 and C6 roots join to form upper trunk. C7 root forms the middle trunk. C8 and T1 roots join to form lower trunk. Each trunk divides into ventral and dorsal divisions. Ventral division of the lower trunk forms medial cord. Dorsal divisions of all the three trunks join to forms posterior cord. Ventral divisions of upper and middle trunk join to form lateral cord. Musculocutaneous nerve (MCN) is the branch from the lateral cord of the brachial plexus. The nerve initially accompanies the axillary artery, pierces the coracobrachialis muscle, and then passes downwards between the biceps brachii and brachialis.

It supplies coracobrachialis, biceps brachii and medial part of brachialis muscles. Below the elbow joint the nerve is continuous as the lateral cutaneous nerve of the forearm [1].

Case presentation:

During routine dissection for first year medical students a variation of the MCN was observed in a 67 year old embalmed male cadaver. The termination of MCN in the left upper limb was variable. After piercing the coracobrachialis muscle, MCN divided into lateral cutaneous nerve of the forearm and gave an accessory branch that joined with median nerve (MN) lateral to the insertion of the coracobrachialis muscle. The ulnar, radial, axillary and remaining course of the MN remained the same.

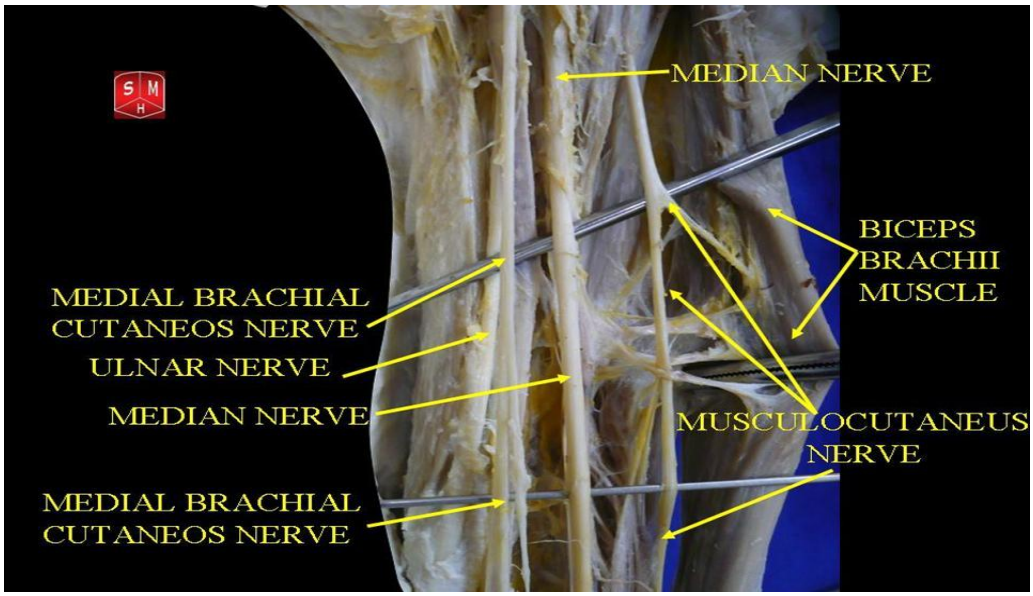

Figure 1: Normal Anatomy of the Arm [With permission:20th edition of Gray's Anatomy (1918)]

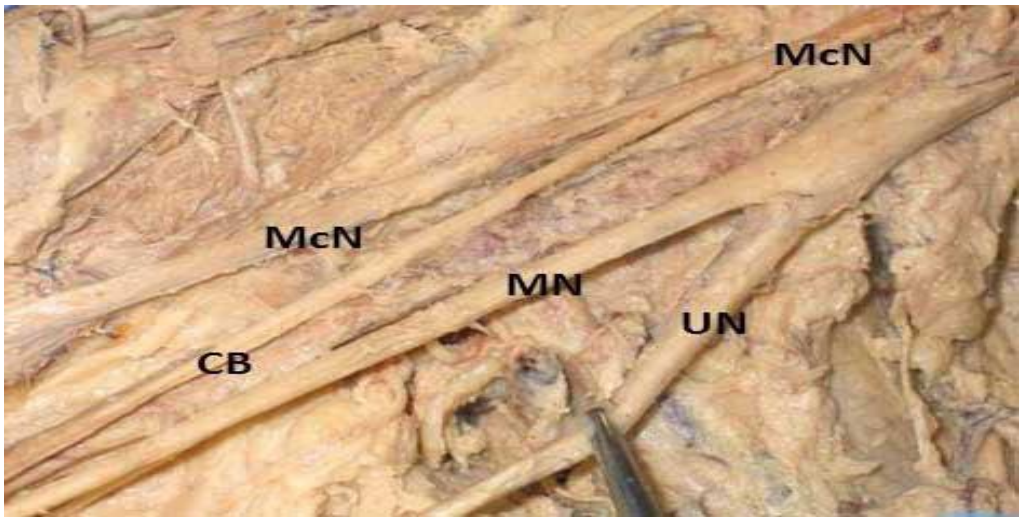

Figure 2: Musculocutaneous nerve (MCN) branch to Median nerve (MN)

DISCUSSION:

The present case reports the anomalous termination of MCN in the left upper limb of a 67 year old male cadaver. Variations of the brachial plexus have been routinely reported in the literature. Venieratos and Anagnostopoulou reported three types of unusual communication between MN and MCN considering the coracobrachialis muscle as the reference point [2]. In type 1, the communication was proximal to the entrance of the MCN into coracobrachialis. In type 2, the communication was distal to the muscle. In type 3, the nerve and the communicating branch did not pierce the muscle. Eglseder and Goldman noticed interconnections between the MCN and MN in 36% of dissections out of 54 cadavers [3]. Loukas and Aqueelah identified 4 different patterns of communication. Out of 129 formaldehyde fixed cadavers 119 communications were found to be present. We were able to identify 4 different patterns of communication. Type I (54 communications, 45%): the communications were proximal to the point of entry of the MCN into the coracobrachialis, Type II (42 communications, 35%): the communications were distal to the point of entry of the MCN into the coracobrachialis, Type III (11 communications, 9%): the MCN did not pierce the coracobrachialis and Type IV (9 communications, 8%): [4]. Venieratos and Anagnostopoulou found types of communications between the MCN and MN based on the sites of communication. Type I: the communication was proximal to the entrance of the musculocutaneous nerve into coracobrachialis; type II: the communication was distal to the muscle; type III: the nerve as well as the communicating branch did not pierce the muscle a study conducted in 79 cadavers [2]. Prasada Rao and Chaudhary reported eight instances of communication from MCN to the MN and bilateral communication in two cadavers [5]. Le Minor classified these variations in to five types. Type 1: no communication between the MN and MCN. Type 2: the fibers of medial root of MN pass through the MCN and join the MN in the middle of the arm. Type 3: fibers of the lateral root of the MN pass through the MCN and after some distance leave it to form lateral root of MN. Type 4: the MCN fibers join the lateral root of the MN and after some distance the MCN arise from the MN. Type 5: The MCN is absent and the entire fibers of MCN pass through lateral root of MN and fibers to the muscles supplied by MCN branch out directly from MN [6].

In the present case report some of the fibers of MN could have been carried by the MCN which later on were passed to MN because the nerve

supply to the muscles of the arm remained as mentioned in the standard books. Such variations also have clinical importance especially in post traumatic evaluations and exploratory innervations of the arm for peripheral nerve repair. The knowledge of the variations of this communication between the musculocutaneous and median nerves in the distal third of the arm is important in the anterior approach for the fracture of the humerus. Clinical implication of this could be that injury of MCN proximal to the anastomotic branch between musculocutaneous and median nerve may lead to unexpected presentation of weakness of forearm flexors and thenar muscles [7].

CONCLUSION:

Knowledge of such variation is a must for surgeons performing intervention in the arm because if the branch from MCN gets damaged it may affect the function of the hand, which may be thought as Carpal tunnel syndrome.

REFERENCES

1. Susan S. Standring, Grays Anatomy, 39th edition, Pg.No-839
2. Venieratos D, Anagnostopoulou S. Classification of communications between the musculocutaneous and median nerves. Clin Anat 1998; 11: 327–331.
3. Eglseder WA Jr, Goldman M. Anatomic variations of the musculocutaneous nerve in the arm. Am J Orthop (Belle Mead NJ). 1997; 26: 777–780.
4. Loukas M, Aqueelah H. Musculocutaneous and median nerve connections within, proximal and distal to the coracobrachialis muscle. Folia Morphol (Warsz). 2005; 64: 101–108.
5. Prasada Rao PV, Chaudhary SC. Communication of the musculocutaneous nerve with the median nerve. East Afr Med J. 2000; 77: 498–503.
6. LeMinor JM. A rare variation of the median and musculocutaneous nerves in man. Arch Anat Histol Embryol. 1990;73: 33–42.
7. Sunderland S. Nerves and nerve injury. In: The median nerve: Anatomical and physiological features: 2nd ed. Churchil Livingstone: Edinburg; 1978. p. 672-7, 691-727.

CASE REPORT

SPONTANEOUS HETEROTOPIC PREGNANCY WITH TUBAL RUPTURE IN A TEENAGER: A CASE REPORT AND LITERATURE REVIEW

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ABSTRACT:
Heterotopic pregnancy is the simultaneous occurrence of intrauterine and ectopic pregnancies. It is rare in spontaneous pregnancies, occurring only in 1 per 30,000. It is a life-threatening condition with diagnostic and therapeutic difficulties. We report a case of a 16 year old with a previous unsafe abortion with a diagnosis of a ruptured ectopic pregnancy. Meticulous Ultra sound assessment resulted in the diagnosis of Heterotopic pregnancy. She had laparatomy and manual vacuum aspiration. Spontaneous heterotopic pregnancy is a life threatening condition especially in poor resource setting like ours with paucity of diagnostic equipment. Clinicians should have a high index of suspicion for heterotopic pregnancy when evaluating young teenage girls with complaints of abdominal pain and amenorrhea. In addition, tackling the unmet need for contraception in our environment, especially emergency contraception will ameliorate the morbidity and mortality associated with this rare condition.

Key Words: Heterotopic Pregnancy, Spontaneous, Teenager, Ultrasound
Submitted: July 2015; Accepted: August 2015

INTRODUCTION:

Heterotopic pregnancy is the simultaneous occurrence of ectopic and intra-uterine gestations [1]. It was first reported in 1708 as an autopsy finding [1]. It is a rare condition occurring in 1 per 30,000 naturally occurring pregnancies, but with an incidence of about 1% in pregnancies following in vitro fertilization IVF [1]. The risk factors include previous tubal surgeries, endometriosis, previous ectopic pregnancy, assisted reproduction technology, previous pelvic inflammatory disease and pelvic adhesions. Moreover, the presence of at least one risk factor for ectopic pregnancy in 71% of women with heterotopic pregnancy had been reported [2].

The treatment options are; surgery, medical treatment and expectant treatment, with the

aim of preserving the intrauterine pregnancy. Methotrexate is usually avoided so as not to compromise the viable intrauterine pregnancy. The use of potassium chloride or hyperosmolar injection, directly injected into the ectopic gestational sac had been reported [3].

The survival rate of intrauterine gestations in heterotopic pregnancies is estimated to be between 66–68%. Moreover, heterotopic intrauterine gestations were 30% less likely to result in live birth than intrauterine pregnancies [3, 4].

The major factor associated with the high maternal morbidity and mortality seen in Heterotopic pregnancy (HP) is delay in diagnosis; because the symptoms of ectopic gestation can be attributed to complications of intrauterine pregnancies especially in asymptomatic patients which may lead to tubal rupture, and it's complications like haemoraghic shock and the requirement for blood transfusions. These complications can also complicate the intra uterine pregnancy component [5]. Transvaginal sonography (TVS) is the imaging modality of choice for the diagnosis of heterotopic pregnancy. The early diagnosis of heterotopic pregnancy by Transvaginal sonography in asymptomatic patients is potentially lifesaving, and enables conservative management options to be considered [5]. We present the case of a 16 year old nullipara with heterotopic pregnancy that was managed with laparatomy, manual vacuum aspiration and blood transfusion.

CASE PRESENTATION:

A 16 year old unmarried nullipara who presented at the emergency unit of our hospital with complains of abdominal pain and swelling of one day duration. Patient had two episodes of fainting attacks prior to presentation, but there was no history of vaginal bleeding, vaginal discharge or urinary symptoms. She was 9 weeks pregnant, from her last menstrual period. It was a spontaneous conception and there was no past history of pelvic inflammatory disease or abdominal surgery. She had an unsafe abortion about a year prior to presentation which was complicated by sepsis. On examination, she was conscious but in obvious painful distress, dehydrated and pale. Her respiratory rate was 22 cycles per minute, pulse rate was 110/min while her blood pressure was 90/60 mmhg. The abdomen was distended, tense and tender making organ palpation difficult. There was a positive fluid thrill. Bowel sounds were normal. Pelvic examination could not be carried out due to the tenderness. She was resuscitated with intravenous fluids and investigated. Trans vaginal ultrasound scan revealed haemoperitonium, a nine week non viable intra uterine pregnancy and an ectopic gestational

sac surrounded by omentum (figure 1). Park cell volume was 19%, WBC and platelet counts were within normal limits. Pregnancy test with Urine was positive. A diagnosis of heterotopic pregnancy with tubal rupture was made. An informed consent was obtained from patient's guardian for an emergency laparotomy. Findings were a ruptured right Ampullary ectopic gestation with hemoperitoneum of about 2 liters and eight (8) weeks sized uterus. Partial salphingectomy, peritoneal lavage and a manual vacuum aspiration of the uterine contents were done. She was transfused with 2 units of blood intra-operatively and additional one unit after surgery. Her postoperative period was uneventful. She was discharged home on the 5th post operative day after she was properly counselled on contraception.

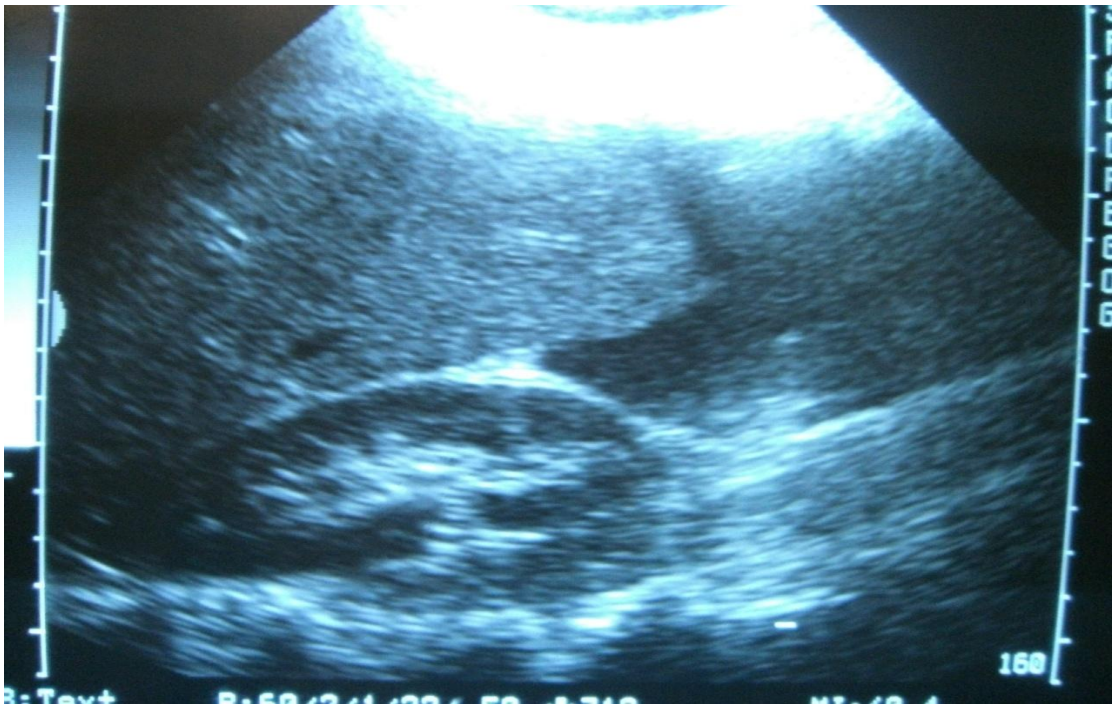

Figure 1: Transvaginal sonography (TVS) showing a non viable intra uterine pregnancy and a rupture ectopic with heamoperitoneum.

DISCUSSION:

Heterotopic pregnancies are thought to be on the increase due to the rising incidence of ectopic pregnancies [1, 6]. The history of a previous unsafe abortion predisposed the patient to having an ectopic pregnancy although there have been reports of spontaneous heterotopic pregnancies occurring in women with no risk factors [2, 7]. Early diagnosis is often Problematic, due to the lack of clearly defined signs and symptoms. Abdominal pain, adnexal mass, peritoneal

irritation and an enlarged uterus were defined as signs and symptoms suspicious of Heterotopic pregnancy [1]. Furthermore, visualization of heart activity in both intrauterine and extrauterine gestation confirms the diagnosis,[1, 5, 6]. Thus, thorough pelvic ultrasonography (USG) in these patients is important, as was done in this case. It is however pertinent to note that the advent of ultrasound (USG) may not have changed the diagnostic ability of Heterotopic over a period of time, this is because it is a rare condition and most patients with Heterotopic pregnancy often present with symptoms of a rupture ectopic component. Thus, a preoperative diagnosis of HP is still a dilemma [1, 8].

The presentation of this patient is in keeping with the pattern of presentation of ectopic pregnancies in Nigeria, which is usually as acute emergencies [9]. It is pertinent to note that heterotopic pregnancies have had successful term deliveries of the intra-uterine component after laparatomy with a success rates of between 66% and 69% reported by previous workers [1, 3]. This may be connected to the improvement in early diagnosis and treatment and close follow- up of patients after in vitro fertilization.

CONCLUSION:

This patient fits the profile of women in the Niger Delta who lack access to contraception and other reproductive health services. Despite an increase in the incidence of ectopic pregnancy in the developed world, early diagnosis facilitated by Ultra sound scan has led to a reduction in morbidity and mortality that is associated with heterotopic pregnancy, hence physicians should exclude heterotopic pregnancy in all women of reproductive age, even in the presence of an intrauterine pregnancy the dictum 'think ectopic' must not be forgotten.

We declared that no competing interests exist.

REFERENCES:

1. Rimpy Tandon, Poonam Goel, Pradip Kumar Saha and Lajya Devi. Spontaneous heterotopic pregnancy with tubal rupture:a case report and review of the literature. Journal of Medical Case Reports.2009, 3:8153 doi: 10.4076/1752-1947-3-8153.
2. Talbot, K., Simpson, R., Price, N., Jackson, S.R. Heterotopic pregnancy. J. Obstet. Gynecol. 2011; 31, 7–12.
3. Tal Shavit, Einat Paz-Shalom, Eylon Lachman, Ofer Fainaru and Adrian Ellenbogen. Unusual case of recurrent heterotopic pregnancy after bilateral salpingectomy and literature review. Reproductive Biomedicine Online 2013;26:59–61 http://dx.doi.org/10.1016/j.rbmo.2012
4. Clayton, H., Schieve, L., Peterson, H., Jameison, D., Reynolds, M., Wright, V. A comparison of heterotopic and intrauterine only pregnancy outcomes

after assisted reproductive technologies in the United States from 1999 to 2002. Fertil. Steril. 2007; 87: 303–309.

5. X. H. Li, Y. Ouyang and G. X. Lu .Value of transvaginal sonography in diagnosing heterotopic pregnancy after in-vitro fertilization with embryo transfer. Ultrasound Obstet Gynecol 2013; 41: 563–569
6. Michał M, Marian M, Marek M, Ewa WO. Heterotopic pregnancy in the absence of risk factors--diagnostics difficulties Ginekol Pol. 2011 Nov; 82(11):866-8.
7. Dassah E.T Odoi AT, Darkey DE, Senaya CM, Djokoto RM Spontaneous heterotopic pregnancy with live infant: report of two cases East Afr Med J. 2008 Dec;85(12):612-5
8. U. A. Menakaya and F. O. Agoreyo Unusual presentation of spontaneous heterotopic pregnancy: A case report International Journal of Biomedical and Health Sciences June 30, 2008; 4 (2)
9. Odewale MA, Afolabi MO. Heterotopic pregnancy: a clinical case report from rural Nigeria Rural and Remote Health 8: 979. (Online) 2008. Available: http://www.rrh.org.au

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