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Can TGF-β Differentiate Fibroblasts and Endothelial Cells into CAFs?: A Research Protocol

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Abstract

Introduction: Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal forms of cancer in Canada. Tumour metastasis contributes to most of the deaths, a process heavily influenced by cancer-associated fibroblasts (CAFs). While the functions of CAFs have been widely researched, such as their metastasis-promoting secretion of growth factors, their origins remain unclear. This research protocol, therefore, seeks to confirm that PDAC cells are capable of differentiating both fibroblasts and endothelial cells into CAFs by secreting transforming growth-factor beta (TGF-β).

Methods: The effects of culturing Hs68 fibroblasts and HMEC-1 endothelial cells in media containing TGF-β will be examined using media supplemented with TGF-β and conditioned media obtained from PANC-1 cells. To confirm these results, TGF-β receptor-inhibited cells will be included also. Proliferation assays, migration assays, RT-qPCR, and western blotting will then be used to determine successful differentiation into CAFs.

Results: It is expected that the presence of TGF-β in culture media will lead to the increased proliferation, migration, and presence of CAF cell markers within the cell culture. The inhibited conditions grown in standard media with the added factor are expected to be comparable to their control groups. The same is expected of the inhibited HMEC-1 cells grown in PANC-1 conditioned media, however the Hs68 culture should more closely resemble its uninhibited condition.

Discussion: The increased results described above for the uninhibited conditions grown in TGF-β-containing media would indicate the following; that this factor is capable of differentiating Hs68 and HMEC-1 cells into CAFs, and that PANC-1 cells are capable of initiating this change. This would be confirmed by the lack of difference between the inhibited versus control conditions; showing that this secreted factor is indeed responsible for these effects.

Conclusion: The results from this protocol will help to solidify fibroblasts and endothelial cells as origins of CAFs, and TGF-β as a CAF-generating factor. By knowing more about their origin, the development of new potential drugs that target the formation of TGF-β is possible. Further directions could include the possibility of in vivo experiments confirming the results of this protocol.

Keywords: cancer-associated fibroblasts; fibroblasts; endothelial cells; transforming growth factor-beta; pancreatic ductal adenocarcinoma; conditioned media

Introduction

Around 90% of all pancreatic cancers are diagnosed as pancreatic ductal adenocarcinomas (PDAC) [1]; known for its highly aggressive desmoplastic biology and dismal prognosis, it is one of the deadliest solid malignancies [1]—resulting in the lowest overall 5-year survival rate of any cancer in Canada [2]. Part of PDAC's aggressive nature can be attributed to its tumour microenvironment (TME), or the surrounding cells, molecules and blood vessels that interact with the cancer cells to either promote or suppress the tumour [3]. Within the PDAC TME, one of the most abundant and important cell types are cancer-associated fibroblasts (CAF) [3]. Their ability to remodel the extracellular matrix (ECM), as well as their secretome, allows them to actively contribute to PDAC progression and regulate the immune response [4].

The functions of CAFs can vary, adopting either tumour-promoting or suppressing capabilities [4]. To this day, the precise origin of CAFs is unclear—due to their heterogeneous populations [5]. Previous research has proposed that a wide array of cell types can transdifferentiate into CAFs through certain signaling pathways [4–6], but this protocol will focus only on fibroblasts and endothelial cells. In normal physiology, fibroblasts maintain the structural integrity of the tissues they reside in, and contribute to the wound healing process by secreting ECM proteins [7]. In contrast, endothelial cells line the blood vessels within the body and act as mediators in exchanges between the bloodstream and surrounding tissues [8]. In the context of PDAC, the transformation of fibroblasts and endothelial cells

into CAFs can be activated by the secretion of several different growth factors from the cancer cells.

Because of the uncertainty surrounding their origin, the determination of CAF-generating factors is imperative to help further understand this cell type. One possible factor to investigate is Transforming growth factor-beta (TGF-β)—a pleiotropic cytokine that plays an important role in cancer progression, promoting tumour proliferation and metastasis through its signaling pathways [9]. Previous research has shown that TGF-β can differentiate both fibroblasts and endothelial cells into CAFs [10,11]. Interestingly, while other growth factors can also differentiate fibroblasts, TGF-β is the only factor shown to differentiate endothelial cells [11–13]. Thus, this protocol aims to validate the role that TGF-β plays in this process, and confirm that PDAC cells can influence this transformation into CAFs through paracrine signaling.

Methods

Cells

The following cell lines would be purchased from American Type Culture Collection (ATCC); Hs68 fibroblasts, HMEC-1 endothelial cells, and PANC-1 pancreatic cancer cells. 293FT cells would be purchased from Invitrogen. All cells would be cultured in Dulbecco's Modified Eagle Medium (DMEM) and 1% L-glutamine except for endothelial cells, which would be grown in MDCB131 and L-glutamine. A supplement of 10% fetal bovine serum (FBS) is added to all cell cultures. Culture conditions of 37°C at 5% CO_2 apply to all cell types.

Conditioned Media Collection

To obtain the conditioned media necessary for this experiment, PANC-1 cells would be seeded in flasks with complete medium. Upon reaching 70% confluency, a media change would be performed, and after 48 hours of incubation, the conditioned media would be collected from the cultures. The conditioned media would then be centrifuged at 1000 x g for ten minutes and filtered through a 0.22 μm-diameter filter.

Lentiviral Vector Creation and Knockdown of TGF-β

Lentiviral knockdown would be used to create TGF-β receptor-inhibited Hs68 and HMEC-1 cells. The construction of the lentiviral vector and subsequent infection of the cells is based on the process outlined in Horie et al. [14]. Briefly, two different micro ribonucleic acid (miRNA) sequences designed to target TGF-β receptors would be ligated separately into shuttle vectors, and then subcloned into the EmGFP-containing lentiviral vector using the BLOCK-iT™ Pol II miR RNAi Expression Vector Kit with EmGFP (Invitrogen). The miRNA sequences used were designed with the BLOCK-iT™ RNAi Designer, with the two sequences targeting either the TGF-β1 or TGF-β2 receptor. 293FT cells would be used to produce the lentiviruses; when 70% confluent, the cells would be transfected in two groups with the two different lentiviral vectors, along with the Lenti-vpak packaging plasmid (OriGene). The media produced by the 293FT cells would then be collected following a 72 hour incubation. To generate TGF-β-inhibited cells, Hs68 and HMEC-1 cultures would be infected with a 50:50 ratio of both types of lentivirus-containing media, for a multiplicity of infection (MOI) of 1 for each cell type. The volume of supernatant required to achieve this MOI would be determined with an enzyme-linked immunosorbent assay (ELISA), and successfully infected cells would be sorted out using fluorescence-activated cell sorting (FACS).

ELISA for Lentivirus Titer

To determine the viral titer of the supernatants, the One-Wash™ Lentivirus Titer Kit (OriGene) would be used following the manufacturer's protocol. Serial dilutions of 1:500 to 1:5000 for each supernatant would be examined as recommended by the kit. After incubating with the detection antibody and streptavidin horseradish peroxidase conjugate, the supernatants would be activated with tetramethylbenzidine and stopped with 1N hydrochloric acid. The absorbance of the supernatants would then be measured with the Multiskan™ FC Microplate Photometer (Thermo Scientific) at 450 nm, and the concentration of p24 within each sample used to calculate the transducing units per mL of the supernatants.

FACS of Hs68 and HMEC-1 Cells

Using FACS, EmGFP-positive Hs68 and HMEC-1 cells would be sorted out for use in future experiments. Approximately 1×10^7 Hs68 and HMEC-1 cells cultured in lentivirus-containing media would be collected and sorted with the S3e Cell Sorter (Bio Rad). EmGFP expression would be detected using the 488 nm laser and the 530/30 nm bandpass filter.

Cell Culture Models

To examine if TGF-β is able to differentiate fibroblasts and endothelial cells into CAFs, an experimental cell culture model would be used. Three different groups each of Hs68 and HMEC-1 cells would be observed under the following conditions; normal cells grown in their corresponding cell culture media, normal cells grown in culture media with added TGF-β, and the TGF-β-inhibited cells grown in culture media with added TGF-β. To further examine whether or not PDAC-secreted TGF-β can initiate this differentiation, the same cell culture model would be applied, using the collected PANC-1 conditioned media instead.

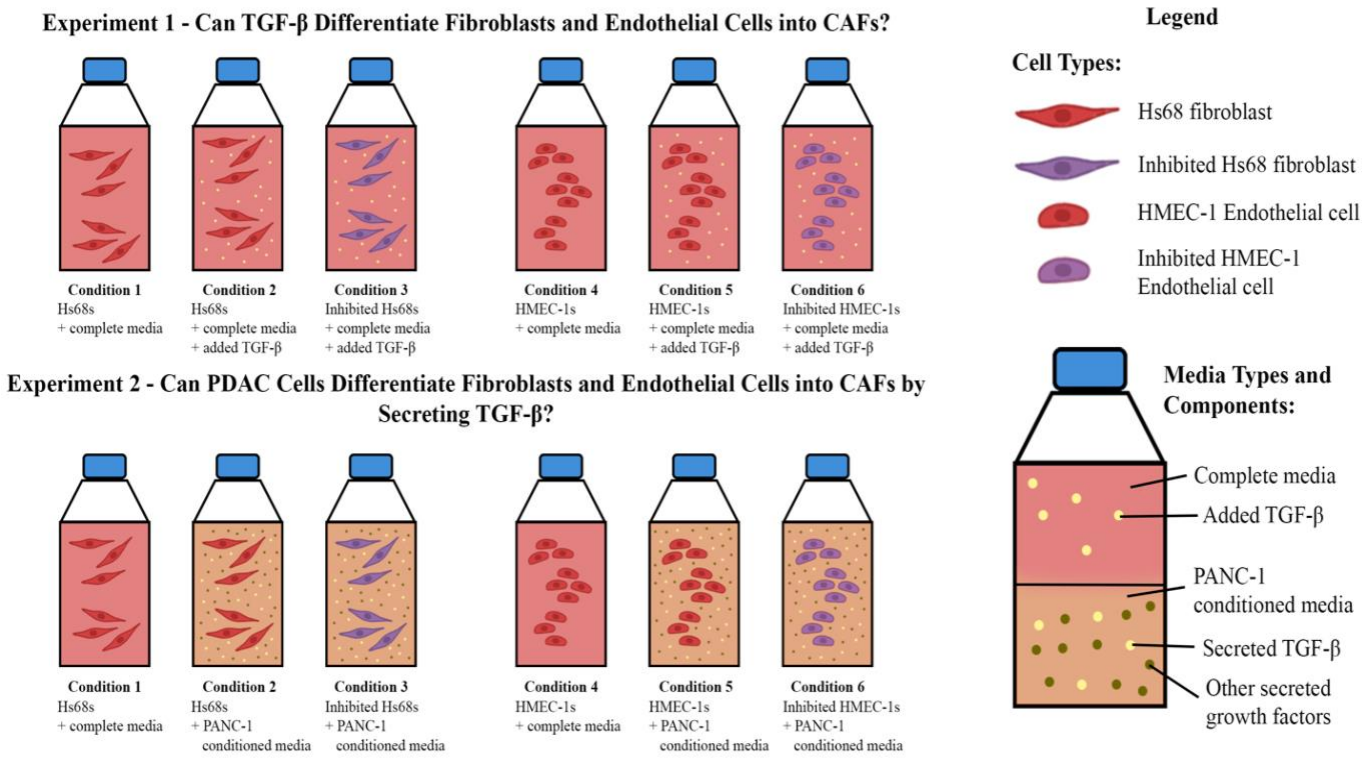

Figure 1. A comparison of the conditions for the two cell culture experiments. This figure was created using Adobe Photoshop CC 2019, version 20.0.4.

Proliferation Assays

The proliferation of the different experimental groups would be compared with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, using the MTT Cell Growth Assay Kit (Roche) as specified in the manufacturer's protocol. After maintaining cultures of Hs68 and HMEC-1 cells for three passages, they would be seeded at 150k per well in 6 well plates containing the media corresponding to their condition in duplicate. Upon reaching 70% confluency, 5mg/mL MTT would be added to each well and the plate would be incubated for four hours, at 37°C and 5% CO_2 in an incubator. Following the addition of the solubilization solution, the plate would be incubated overnight under the same conditions. The absorbance of the samples would then be read at 600 nm using the Multiskan™.

Migration Assays

To assess the migratory ability of each condition, the ECM510 QCM Chemotaxis Cell Migration Assay (Sigma-Aldrich) would be used according to the manufacturer's protocol. Cells would travel through the membrane in the top chamber, filled with each condition's serum-starved media, to the bottom chamber containing their standard media. Passage three Hs68 and HMEC-1 cells would be serum-starved for 18-24 hours, before being plated at 25k cells per top chamber of the plate. The migration of each condition across the membrane would be measured by detaching and lysing the cells in the bottom chamber, adding CyQuant GR dye, and then reading the absorbance of each well at 520 nm with the Multiskan™.

Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

RT-qPCR would be done to determine the presence or absence of CAFs in each of the Hs68 and HMEC-1 conditions by looking at their messenger RNA (mRNA) expression of specific surface markers. The biomarkers chosen to be studied are the CAF markers fibroblast activation protein (FAP) and alpha smooth muscle actin (αSMA), the fibroblast marker fibroblast-specific protein 1 (FSP-1) and the endothelial cell marker platelet and endothelial cell adhesion molecule 1 (PECAM-1). Using the $\Delta\Delta C_t$ method in line with Horie et al. [14], the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) would be used to normalize the mRNA expression of these biomarkers. RT-qPCR analysis would be conducted following thirty days of culture, after which the RNA from the cells would be isolated with the RNeasy Plus Mini Kit (Qiagen). Genomic deoxyribonucleic acid (gDNA) digestion, primer annealing and subsequent reverse transcription of the mRNA would be done with the SuperScript IV VILO Master Mix (Invitrogen). Finally, qPCR amplification of the produced cDNA would be done with the PowerTrack SYBR

Green Master Mix kit (Applied Biosystems). All forward and reverse primers would be purchased from OriGene.

Cell Lysis

To obtain the cell lysates necessary for western blotting, radio-immunoprecipitation assay lysis buffer (Sigma-Aldrich) would be added to the Hs68 and HMEC-1 conditions, after an ice-cold 1X phosphate-buffered saline wash. The cells would then be scraped, collected in microcentrifuge tubes and agitated for thirty minutes at 4°C in a microcentrifuge tube shaker. Cell lysates would be collected after centrifuging the tubes at 16 000 x g for twenty minutes at 4°C, and their total protein concentrations estimated with the Pierce™ Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Scientific).

BCA Assay

Following the manufacturer's protocol, 25 µL of each lysate and standard would be measured in duplicate on a 96 well plate. After incubating with the Working Reagent for thirty minutes at 37°C, the absorbance of the samples would be read at 562 nm using the Multiskan™. From these results, aliquots of the different lysates at equal total protein concentration would be made in preparation for the western blot.

Western Blot

To further confirm the results of the RT-qPCR, western blotting would be done to check the protein expression levels of the different Hs68 and HMEC-1 conditions. The same cell surface markers used in the RT-qPCR experiments would be examined in this section. The western blotting in this protocol would follow OriGene and Sigma-Aldrich's protocols as outlined on the resource pages of their websites. The cell lysates of the Hs68 and HMEC-1 conditions would be run on two separate mPAGE® 4-12% Bis-Tris Precast Gels (Millipore) at 170 V; one gel for the detection of the CAF markers, and the other for the fibroblast and endothelial cell markers. The two gels would then be transferred onto Immobilon®-FL membranes (Sigma-Aldrich) at 100 V. The membranes would be blocked for one hour with tris-buffered saline (TBS) and 5% nonfat dry milk, then incubated overnight at 4°C on a shaker with the primary antibodies in TBS with Tween® 20 (TBST) and milk. Lastly, the membranes would be incubated with the fluorescent secondary antibodies in TBST with milk for one hour on a shaker. The fluorescence would then be measured with the iBright™ FL1500 Imaging System (Invitrogen) and its lasers at 488, 647 and 555 nm. All positive control lysates for the proteins of interest and the GAPDH loading control would be purchased from OriGene, and all antibodies would be purchased from Invitrogen.

Table 1. Catalog numbers and concentrations of the antibodies used in the western blotting experiments

	Gel 1: CAF+ Markers		**Gel 2: Fibroblast+ and Endothelial+ Markers**	
	FAP	**αSMA**	**FSP-1**	**PECAM-1**
Primary Antibody (PA)	#PA5-99313	#14-9760-82	#PA5-18601	#BMS137
PA Concentration (µg/mL)	1	5	0.1	5
Secondary Antibody (SA)	#A32731	#A-21241	#A32816	#A-21241
SA Concentration (µg/mL)	0.2	0.2	0.2	0.2

Results

Effects of TGF-β on Fibroblasts and Endothelial Cells

Firstly, an MTT assay would be used to assess the proliferation of each of the conditions. It is expected that the non-inhibited conditions grown in TGF-β-containing media will have increased proliferation compared to the controls, and the inhibited conditions are expected to have similar proliferation. The presence of CAFs in the culture conditions would then be examined with a chemotaxis cell migration assay. Compared to the controls, uninhibited conditions grown in TGF-β-containing media are expected to have the greatest number of migrated cells. The Hs68 condition would have a higher amount than the HMEC-1 condition, as studies have shown that fibroblasts have a higher migration rate than endothelial cells [15,16]. The inhibited conditions are expected to have a similar number of migrated cells as the controls. Overall, the proliferation and migration rate of both the Hs68 and HMEC-1 conditions are expected to increase in the presence of TGF-β.

To confirm the presence of CAFs in the cultures, levels of FAP, αSMA, FSP-1 and PECAM-1 mRNA expression would be measured with the use of RT-qPCR. Both the Hs68 control and inhibited conditions are expected to have high levels of FSP-1 mRNA, but not the CAF markers FAP and αSMA, and not PECAM-1. The uninhibited Hs68 condition grown in TGF-β-containing media should have high levels of FAP and αSMA expression, with some FSP-1 and no PECAM-1 expression. The results for the HMEC-1 cell conditions should follow the same theme as for the fibroblasts, with PECAM-1 expression being present instead of FSP-1. CAF presence would also be

confirmed with western blotting; the amount of FAP, αSMA, FSP-1 and PECAM-1 present in cell lysate would be evaluated for all of the cultures. As for the RT-qPCR experiment, the uninhibited conditions with TGF-β present are expected to have high levels of FAP and αSMA, with some of either FSP-1 or PECAM-1, depending on the condition cell type. The controls and inhibited conditions should have either high FSP-1 or PECAM-1 content, but no FAP and αSMA. These two experiments should demonstrate that the number of cells expressing CAF markers increases with the addition of TGF-β.

Effects of Pancreatic Cancer Cell Conditioned Media on Fibroblasts and Endothelial Cells

As in the first MTT assay, it is expected that the non-inhibited conditions will have an increased proliferation compared to the controls. The inhibited conditions however would produce different results; the HMEC-1 condition would have a similar proliferation to its control, but the Hs68 condition is expected to have a higher proliferation than its control, and a lower proliferation than the uninhibited condition. This second migration assay would reveal that, again, the uninhibited conditions would have the highest number of migrated cells compared to their controls when grown in PANC-1 conditioned media–the Hs68 condition being higher than the HMEC-1 condition. Following the theme of the last experiment, the inhibited HMEC-1 condition would have similar results to its control, but the inhibited Hs68 condition's migratory ability would be in between the control and the uninhibited condition. Altogether, growing both cell types in PANC-1 conditioned media should lead to an increase in proliferation and migration.

As in the previous part, analysis with RT-qPCR would reveal that the Hs68 control would have high amounts of FSP-1, with no FAP, αSMA or PECAM-1 mRNA expression. The uninhibited Hs68 condition would follow the previous experiment's results as well, having high amounts of FAP and αSMA expression, with some FSP-1 and no PECAM-1. The HMEC-1 control and uninhibited conditions would follow the same formula, with PECAM-1 expression instead of FSP-1 being present. The difference lies in the Hs68 inhibited condition; while the HMEC-1 condition is expected to have the same expression profile as it's control, the Hs68 condition's expression profile would instead be similar to the uninhibited condition–with more FSP-1 expression than FAP and αSMA. Western blotting for FAP, αSMA, FSP-1 and PECAM-1 would then show similar results to the RT-qPCR analysis; high amounts of only FSP-1 or PECAM-1 for the control conditions, high amounts of FAP and αSMA and some FSP-1 or PECAM-1 for the uninhibited conditions, only PECAM-1 expression for the inhibited HMEC-1 condition, and high FSP-1 with some FAP and αSMA expression for the inhibited Hs68 condition. In summary, the growth of both cell types in PANC-1 conditioned media is expected to produce cells with CAF markers.

Discussion

With respect to the uninhibited cultures grown in TGF-β-containing media; the increased proliferation, migration, and presence of αSMA and FAP compared to the controls would indicate that TGF-β is capable of differentiating both Hs68 and HMEC-1 cells into CAFs. This is due to CAFs having a higher proliferation and migration rate than fibroblasts and endothelial cells [17–19], and being the only cell type of the three to express FAP and αSMA [20]. Another indicator that TGF-β is responsible for the generation of these CAFs, would be the similar results between the controls and inhibited conditions grown in TGF-β-containing media. As the inhibited conditions are unable to express TGF-β receptors, they should be unaffected by the presence of TGF-β and thus unable to differentiate into CAFs.

For the uninhibited cultures grown in PANC-1 conditioned media, the same increased results as above would indicate that PANC-1 cells are able to differentiate fibroblasts and endothelial cells into CAFs through paracrine signaling. The similar results between the inhibited HMEC-1 condition to its control would confirm this idea, as it would demonstrate that TGF-β is present in the conditioned media and is therefore responsible for the presence of CAFs in the uninhibited condition. Conversely, if the inhibited HMEC-1 condition's results align more with its uninhibited condition—as is expected of the Hs68 fibroblast conditions—it's possible that there is another factor able to differentiate endothelial cells into CAFs.

The outcome described above is not accounted for in this protocol, as there is a lack of studies on the endothelial origins of CAFs in the context of PDAC. If the need arises, future experiments would be necessary to determine which other component(s) of PANC-1 conditioned media are capable of generating CAFs. Another limitation is the absence of any in vivo experiments in this protocol, as well as the general lack of in vivo experiments related to this topic. The results expected in vitro would be more difficult to evaluate in vivo, and the complexity within the organism's body could lead to unexpected outcomes.

Conclusions

In summary, the primary intent of this protocol is to determine if PDAC cells are able to differentiate fibroblasts and endothelial cells into CAFs by secreting TGF-β. By proving that TGF-β can influence CAF creation, this study could encourage the development of PDAC treatments involving the inhibition of TGF-β receptors. This protocol could also cement fibroblasts and endothelial cells as potential origins for CAFs, and has the possibility of discovering that TGF-β is not the only growth factor capable of generating CAFs from endothelial cells. To build on the results of this protocol, future studies involving in vitro models examining the differentiation of endothelial cells and fibroblasts into CAFs could be conducted.

List of Abbreviations Used
ATCC: American type culture collection
BCA: bicinchoninic acid
CAF: cancer-associated fibroblast
DMEM: Dulbecco's modified eagle medium
ECM: extracellular matrix
ELISA: enzyme-linked immunosorbent assay
FACS: fluorescence-activated cell sorting
FAP: fibroblast activation protein
FBS: fetal bovine serum
FSP-1: fibroblast-specific protein 1
GAPDH: glyceraldehyde 3-phosphate dehydrogenase
MOI: multiplicity of infection
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
PDAC: pancreatic ductal adenocarcinoma
PECAM-1:platelet and endothelial cell adhesion molecule 1
RT-qPCR: reverse transcription-quantitative polymerase chain reaction
TBS: tris-buffered saline
TBST: tris-buffered saline with Tween® 20
TGF-β: transforming growth factor-beta
TME: tumour microenvironment
gDNA: genomic deoxyribonucleic acid
mRNA: messenger ribonucleic acid
miRNA: micro ribonucleic acid
αSMA: alpha smooth muscle actin

Conflicts of Interest
The author declares that they have no conflict of interests.

Ethics Approval and/or Participant Consent
This study did not require ethics approval and/or participant consent.

Authors' Contributions
MET: made substantial contributions to the design of the study, the collection of data as well as interpretation and analysis of the data, revised the manuscript critically, and gave final approval of the version to be published.

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