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ORIGINAL ARTICLE

Vasculogenic Mimicry in Clinically Non-functioning Pituitary Adenomas: a Histologic Study

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Abstract The term "vasculogenic mimicry" (VM) refers to the phenomenon in which vascular-like channels, which are not lined by endothelial cells, are formed in tumors. Since its discovery in 1999, it has been observed in several tumor types and is proposed to provide blood perfusion to tumors in absence of co-apted or neo-angiogenic blood vessels. Pituitary tumors are generally slow growing, benign adenomas which are less vascularized than the normal pituitary gland. To date, VM in pituitary adenomas has not been described. In this histological study, we assessed the presence of VM in a series of surgically resected clinically non-functioning pituitary adenomas (NFPAs) using CD34 and Periodic Acid-Schiff (PAS) double staining. To identify VM, slides were assessed for the presence of CD34-negative and PAS-positive channels indicating that they were not lined by endothelial cells. The histological staining pattern suggestive of VM was noted in 22/49 (44.9%) of the specimens studied. VM was observed in both recurring and non-recurring NFPAs. The incidence of VM present varied from case to case and within groups. There was no association between the presence of VM and gender, tumor size, Ki-67 index, recurrence or cavernous sinus invasion. VM was not noted in cases of non-tumorous pituitaries. Our findings suggest the existence of a complementary perfusion system in pituitary adenomas, implying potential clinical implications with respect to response to therapy and clinical course. Further research is warranted to confirm the presence of VM in pituitary adenomas to elucidate its clinical relevance in patients diagnosed with a pituitary adenoma.

Keywords Biomarkers · Pathology · Pituitary adenoma · Microvascularity · Vasculogenic mimicry

Abbreviations

DAB	Diaminobenzidine tetrahydrate
GBM	Glioblastoma multiforme
H&E	Hematoxylin-eosin
NFPA	Clinically non-functioning pituitary adenomas
PAS	Periodic Acid-Schiff
PBS	Phosphate buffered saline
VM	Vasculogenic mimicry
WHO	World Health Organization

Introduction

Tumor development, growth and metastasis, require blood supply. Several types of mechanisms of vessel formation, including non-angiogenic tumor vascularization, have been demonstrated in normal and neoplastic tissues. *Angiogenesis* describes new vessel formation from pre-existing vessels while *vasculogenesis* is the term used to describe the formation of new vessels de novo. In 1999, Maniotis et al. identified vascular channels lined by tumor cells and not endothelial

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cells in both primary and metastatic melanoma tissues [1]. These vascular channels were shown to form a functional network allowing for tissue perfusion with blood in vitro. Subsequently, "vasculogenic mimicry" (VM), as this phenomenon was termed, has been proposed as an additional mechanism for neoplastic blood perfusion. In addition to melanoma, VM has also been reported in glioma, glioblastoma multiforme (GBM), gastric, and breast carcinomas as well as several other tumor types [2–10].

The presence of VM has important clinical implications. A recent systematic review and meta-analysis of the literature has identified a significant relationship between the presence of VM and low 5-year survival rates in eight types of malignant neoplasms [11]. The same study also concluded that VM contributes to the development of metastasis in melanoma by providing a blood and nutrient supply to tumor cells, thereby enhancing tumor growth, as well as by providing a functional network of channels that may facilitate cell migration to distant sites independent of tumor angiogenesis [1, 11]. The presence of VM may also account for the variability in the effectiveness of anti-angiogenic therapies [12]. It is thought that VM networks are an additional pathway for neoplastic perfusion which are also less likely to respond to anti-angiogenic treatment [5, 12, 13]. Tumors may well maintain perfusion, nutrient delivery, and consequently cell proliferation and growth despite these targeted treatment strategies. It was also suggested that channels not bound by endothelial cells but directly by tumor cells in GBM confers a degree of resistance to radiotherapy; an important finding given that radiation is a mainstay treatment of such a malignancy [8, 14]. According to this data, VM is a potential prognostic and therapeutic biomarker in oncology.

Assessment of VM is not conclusive because in case of intratumoral hemorrhage, as it is often found in post-surgical specimens, the presence of blood affects the analysis of the microvascular networks, since blood cells cannot be easily identified surrounded by endothelial cells or PAS-positive matrix. That is, a matrix consisting of polysaccharides, glycoproteins, and glycolipids, among other compounds, but lacking a lining of endothelial cells that is noted in blood vessels [15]. In order to identify and differentiate between endothelial lined blood vessels, hemorrhages and subendothelial PAS-positive matrix that make up VM, the most useful method to employ is the double staining technique using CD34 and PAS which have been used by various researchers since its inception [1, 3, 5, 6].

Pituitary tumors are generally slow growing, benign adenomas and have a high prevalence in the population [16]. Treatment options are generally limited to surgical resection, medication such as long acting somatostatin analogs and dopamine agonists, and/or radiotherapy. Invasion and recurrence are important features in the assessment of prognosis. It is well known that pituitary adenomas are less vascularized than the normal pituitary gland [17]. To date, VM has not been investigated in the pituitary tissue.

We herein conducted an exploratory study to evaluate the histological presence of VM in the normal pituitary gland as well as in clinically non-functioning pituitary adenomas (NFPAs).

Materials and Methods

In this study, we examined 49 cases of surgically resected clinically non-functioning pituitary adenomas (NFPAs) that were randomly selected from the pathology database at St. Michael's Hospital (Toronto, Ontario, Canada). We focused on NFPAs because they account for 50% of all pituitary adenomas and pose a real challenge for clinicians since they show a diverse phenotype, do not result in excess pituitary hormone production, are usually slow to produce symptoms and tend to grow large before being discovered. Specimens were excluded if intratumoral hemorrhage was noted in imaging and/or pathological reports in order to avoid confounding factors on the evaluation regarding the presence of non-endothelial vascular channels. Specimens were divided into two groups (recurring and non-recurring) based on the clinical and pathological data. Recurrence was evaluated according to the radiological follow-up and the consecutive necessity to repeat surgical resection. Included in our sample were 19 recurring NFPAs (age range: 25–58; 9 males/10 females; mean age: 48 +/− 2.3 years) and 30 non-recurring NFPAs (age range: 39–75; 14 males/16 females; mean age: 55 +/− 2.2 years). All tumors were macroadenomas (size >10 mm). Also included in the study were 10 surgically resected and 5 autopsy-obtained non-tumorous pituitaries from patients with no endocrine diseases and no hormone treatment (age range: 27–82; 7 males: 8 females; mean age: 53 +/− 2.3 years). Since VM is the result of the formation of vascular-like channels formed directly by neoplastic cells rather than endothelial cells, normal pituitary gland served as negative controls.

Specimens were formalin-fixed, dehydrated in graded ethanol and paraffin-embedded. Sections of 4 μm thickness were stained with hematoxylin-eosin (H&E), PAS, and the Gordon-Sweet silver method for the demonstration of reticulin fibers. Immunohistochemical analysis using antisera directed toward the adenohypophysial hormones, including the alpha chain of the glycoprotein hormones (alpha subunit) and synaptophysin [18]. Immunohistochemical investigation was used to accurately classify the tumors and assess their apparent endocrine activity. Tumors were classified according to the criteria defined by the World Health Organization (WHO) [19, 20].

Double Staining Procedure

Double staining for CD34-PAS was undertaken using paraffin embedded sections. Sections of 4 μm thickness were deparaffinized and rehydrated. Subsequent to antigen retrieval by microwaving in 0.1 mM sodium citrate buffer (pH 6.0) at 90 °C for 15 min followed by a cooling period of 30 min at room temperature, the sections were rinsed in PBS and then blocked for 10 min using serum-free protein blocker (DAKO North America Inc., Carpinteria, CA). Immunostaining for CD34 primary antibody was achieved by the streptavidin-biotin-peroxidase complex protocol using the LSAB+ Kit (DAKO, Carpenteria, CA) and a CD34 mouse monoclonal antibody (Santa Cruz Biotechnology, Dallas, Texas, USA; dilution: 1:150). Sections were exposed to primary antibody for 30 min at 38 °C; diaminobenzidine tetrahydrate (DAB) chromogen for 10 min and rinsed in tap water. Following CD34 immunostaining, sections were stained with PAS to highlight the subendothelial matrix-associated vascular channels in the pituitary adenomas. Sections were then counterstained with Mayer's hematoxylin for 1 min and cover slipped using permanent mounting medium. To visualize the CD34 and PAS staining, sections were examined under light microscopy.

In an subset of cases (25) taken from our original sample, we also investigated CD31- PAS staining to determine if any differences existed between CD34 and CD31 staining patterns. Immunostaining for CD31 primary antibody was achieved by the streptavidin-biotin-peroxidase complex protocol using the LSAB+ Kit (DAKO, Carpenteria, CA) and a CD31 mouse monoclonal antibody (Santa Cruz Biotechnology, Dallas, Texas, USA; dilution: 1:100). Sections were exposed to primary antibody for 30 min at 38 °C; chromogen (DAB) for 10 min and rinsed in tap water. Following CD31 immunostaining, sections were stained with PAS following the protocol as described above. The CD31-PAS stained cases were examined under light microscopy.

The stained slides were analyzed independently by three of the authors (ADI, FR, KK) in a blinded manner applying the evaluation criteria of Folberg et al. [21]. All sections were evaluated both under low magnification (100×) and high magnification (400×) in order to properly visualize the double staining patterns as well as the PAS-positive and CD34 negative vessels.

Statistical Analysis

The statistical analysis was performed with SPSS v.13.0. Associations between VM and clinical data were determined by the Z-test calculated for two population proportions using a 2-tailed hypothesis. The statistical significance threshold was set at $p = 0.05$.

Results

Immunopositivity for CD34 demonstrated endothelial cells lining blood vessels, which were clearly localized in all 49 cases of NFPAs. The PAS- positive pattern, which stained subendothelial matrix, was also clearly visible in all cases. In order to identify VM, slides were assessed for the presence of CD34-negative and PAS-positive channels containing erythrocytes indicating that they were not lined by any endothelial cells. In the specimens, the presence of VM was suggestive in 22 of the 49 cases (44.9%) studied (Table 1). Vasculogenic mimicry was observed in both the recurring and non-recurring NFPAs studied but the amount of VM varied both from case to case and within groups (Figs. 1, 2 and 3). No statistically significant difference was found between recurring and non-recurring adenomas, although this result may be related to the limited sample size. However, there was a significant difference with respect to the presence of VM when adenoma groups were compared to the non-tumorous pituitaries. CD31-PAS staining was also investigated in 25 cases taken randomly from our original sample. We were able to demonstrate areas in which there was PAS positive and CD31 negative pockets that contained blood cells. They were similar to the CD34-PAS staining. There was no significant difference between the two staining procedures. We preferred to use the CD34-PAS staining method which provided more conclusive results and it also confirmed the results obtained by other publications who used CD34-PAS staining to study VM [1, 3, 5, 6] (Fig. 4).

No statistically significant correlation was found between the presence of VM within each group and the gender of the patients. We also analyzed VM and age and found that in the 22 cases studied where VM was identified, 14 cases (64%) were in the age range between 52 and 59 years. Also, in the 27

Table 1 Vasculogenic mimicry in nonfunctioning adenomas

Nonfunctioning adenoma type	N	M:F	Age range	Mean age	VM positive	VM negative	Z-score	*P* value
Recurring	19	9:10	25–58	48	10	9	0.32	0.75[a]
Non-recurring	30	14:16	39–75	55	12	18	-1.55	0.12[a]
Non-tumorous pituitary gland	15	7:8	27–82	53	0	15	-5.48	0

[a] Z-test calculated for 2 population proportion; 2-tailed hypothesis

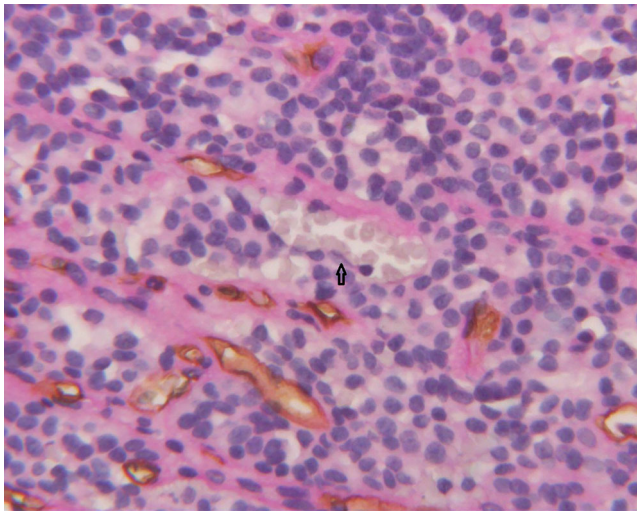

Fig. 1 Clinically non-functioning pituitary adenoma stained with CD34 and PAS. Note the CD34 negative and PAS positive matrix-rich vascular-like channel (*arrowhead*) filled with red blood cells and absent of endothelial cells. CD34 positive blood vessels are also noted. Original magnification: 250X

cases where VM was not identified, 12 cases (44%) were in the age range between 51 and 57 years, and 81% (22/27) of the VM negative cases studied were in the age range between 31 and 57 years.

The NFPAs investigated in our study were all radiologically classified as macroadenomas (size >10 mm). Ki-67 nuclear labeling index, which is an indicator of cell proliferation, was generally low (1–3%) in both the recurring and non-recurring groups. No correlation was found between the presence of VM within the cases studied, Ki-67 index or tumor size.

To determine whether VM was present in non-tumorous pituitary tissue, both surgically removed and autopsy obtained

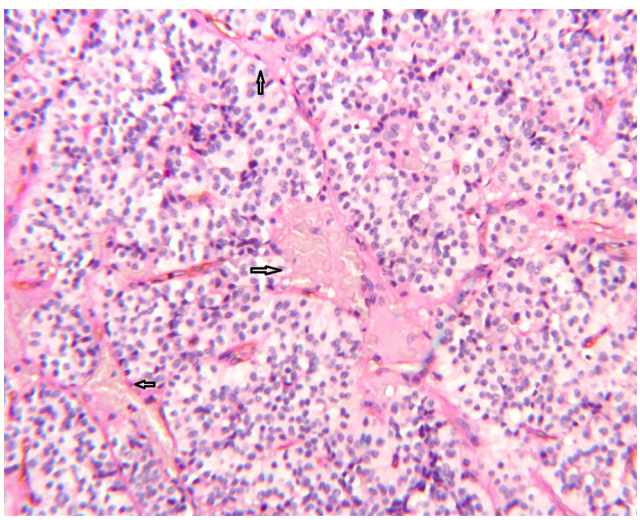

Fig. 2 Clinically non-functioning pituitary adenoma stained with CD34 and PAS. Many matrix-rich vascular-like channels containing red blood cells (*arrowhead*) that are positive for PAS and negative for CD34. Note the absence of blood vessels to this area in the tumor indicating that VM is supplying blood to the tumor. Original magnification: 100X

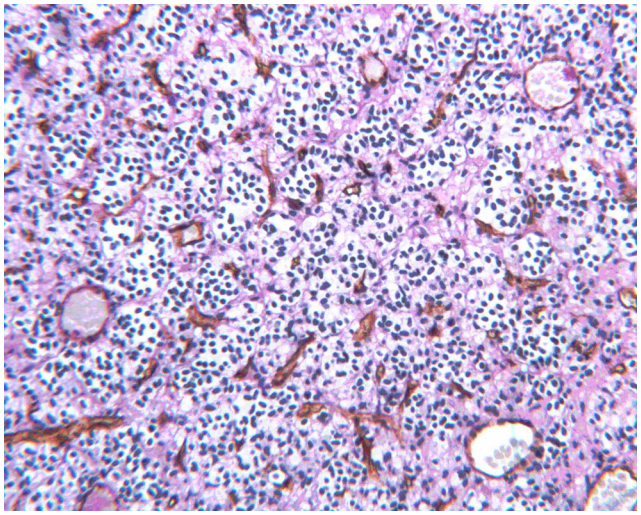

Fig. 3 Non-tumorous pituitary adenohypophysis showing several blood vessels. VM is not present in the non-tumorous pituitary. Original magnification: 100X

pituitaries were double stained as well. As expected, VM was not demonstrated in any of the non-tumorous pituitaries.

Both recurring and non-recurring groups had many similar clinical characteristics including visual disturbances, hypopituitarism, menstrual irregularities in females and impotence in males, and loss of libido.

In both groups (recurring and non-recurring NFPAs), we also looked for a potential correlation between cavernous sinus invasion and VM, but no significant correlation was found.

Discussion

Vasculogenesis is the de novo formation of endothelial cells from mesodermal cell precursors (angioblasts), which lead to

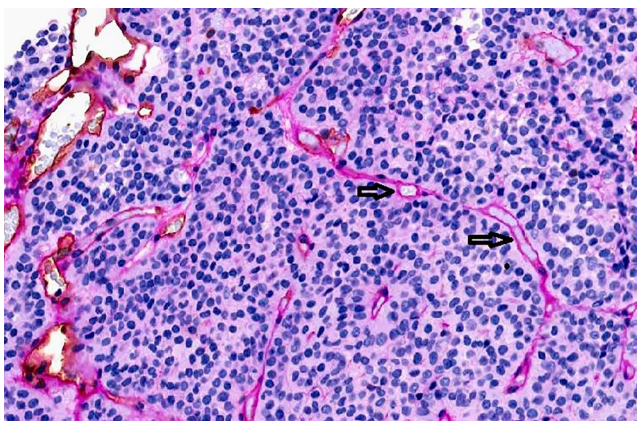

Fig. 4 CD31-PAS staining was also investigated in samples. Areas in which there was PAS positive and CD31 negative pockets that contained blood cells were also noted (*arrows*). Results from the CD31-PAS double staining were similar to the CD34-PAS staining. Original magnification: 200X

the formation of a network of vessels. This process is related to the formation of blood vessels during embryonal development [22–24]. Angiogenesis, on the other hand, is the formation or development of new blood vessels resulting from the proliferation of mature endothelial cells of pre-existing vessels. Angiogenesis is a normal process in growth and development but it has been correlated with tumor progression and poor prognosis in various tumor types [22, 25]. There is, however, evidence that tumor growth and progression can also occur in the absence of angiogenesis [26, 27]. In such situations, it has been suggested that non-angiogenic vascularization may play an important role in tumor growth and progression [12, 26–28]. Vascular co-option is a mechanism whereby tumors attain blood supply by seizing (co-opt) the existing vasculature and the tumor cells proceed to migrate along the vessels of the host organ [28, 29]. Another non-angiogenic mechanism is known as intussusception or splitting angiogenesis. This type of vessel formation involves extension of a capillary wall into the lumen to split a single vessel in two [30, 31]. This is a particularly important process because it increases the number of capillaries without a corresponding increase in the number of endothelial cells [30, 31]. To date, and to our knowledge, neither one of these two mechanisms have been described in pituitary tumors. VM is a third mechanism in which there is the generation of endothelial cell-independent microcirculation within tumors. VM channels are formed via a PAS-positive matrix but not by endothelial cells. There is evidence that VM is present in various tumor types including melanoma, hepatocellular carcinoma, gastrointestinal stromal tumors and ovarian and prostate carcinomas and it has also been postulated that tumors whereby VM is present are more aggressive and have a worse prognosis than tumors without the presence of VM [32–38].

To our knowledge, this is the first report to demonstrate VM in cases of surgically resected NFPAs based on our double staining results. The presence of VM in the cases studied was not indicative of tumor recurrence, cavernous sinus invasion, gender, and tumor Ki-67 cell proliferative index. These results indicate that VM in pituitary adenomas may be distinct from endothelial lined vessels. In regards to NFPAs, it was also demonstrated by Ferreira et al., that there is no statistically significant correlation between a positive immunohistochemistry for Ki-67 and tumor size as with other neoplasms [39]. However, a large proportion of VM-positive specimens were resected from patients between the ages of 52–57. This is not a surprising finding since one of the clinical features of NFPAs at diagnosis is the higher frequency among the older age group. This can be explained, in part, to the fact that NFPAs do not produce excess pituitary hormones and they usually do not produce symptoms until they have grown to considerable size (macroadenoma) before being discovered. The lack of indicators to facilitate early diagnosis leads to mass effects which include pressure on the normal pituitary gland causing hypopituitarism, and on the optic nerve/chiasm causing visual defects.

Several studies have demonstrated a correlation between the presence of VM and increased tumor aggressiveness [5, 6, 8, 10]. In light of the findings presented by these studies, our investigations should have also found correlation between more aggressive adenoma behaviour and the presence of VM, however, our studies found no correlation between VM and recurrence, tumor proliferative index, or cavernous sinus invasion. This lack of significant association may be attributable to a small sample size and insufficient data. The clinical follow-up of the patients in our dataset has not been long enough to determine eventual recurrences or clinical aggressiveness in a delayed fashion, and no transformation in pituitary carcinomas has been recorded. Therefore, our findings cannot exclude the role of VM as a potential biomarker of clinical aggressiveness.

Furthermore, our study sample comprised only macroadenomas and no attempts were made to either assess the presence of VM in microadenomas or correlate VM with tumor size.

The growth and spread of a tumor is due to several factors including angiogenesis and vasculogenesis and the degree of vascularization in tumors correlates with tumor proliferation and expansion [40]. In contrast to other tumor types, it has been shown that pituitary adenomas are less vascularized than their non-tumorous pituitaries [41–43]. A study by Turner et al. on angiogenesis in pituitary adenomas and normal pituitary gland suggested that angiogenic inhibitors may be present in pituitary tumors that play a role in their behavior [42]. Tumors require abundant blood supply to provide the tumor cells with sufficient nutrients and oxygen for their survival and growth. Therefore, it is possible to speculate that other factors (i.e. VM) may modulate the blood supply in pituitary adenomas. In other words, since the level of vascularization is lower in pituitary adenomas, VM could create a collateral perfusion path to maintain adequate blood supply to the tumor and prevent necrosis. In this regard, VM would complement the existing vascular system thereby showing that pituitary tumor cells have more than one way of surviving. These speculations could be further investigated by looking for correlations between VM and pituitary apoplexy, for example, or by analyzing the role of pericytes to support the neoplastic microvascular networks. VM provides the functional plasticity of tumor cells by forming de novo vasculogenic-like networks that have the ability to provide tumor perfusion network for growth and proliferation of tumors.

The presence of VM in pituitary adenomas is also an important finding regarding treatment. Currently, medications such as long acting somatostatin analogs and dopamine agonists are available as a form of treatment for some pituitary adenomas. Liu et al. [9] and Ruffini et al. [44] demonstrated the ability to inhibit VM in gastric cancer using dihydroeffusol

and melanoma cells using integrin inhibitors, respectively. If it is possible to similarly inhibit VM in pituitary adenomas, patients may receive more benefit from medical therapy. However, it is difficult to speculate what effect VM inhibition may have on clinical course without first developing a greater understanding of how VM influences tumor behaviour. Furthermore, more investigations would be required in order to establish which medical therapies would have VM-inhibitory effects. While Liu et al. and Ruffini et al. [9, 44] were able to demonstrate successful VM inhibition, an earlier study by Liu et al. [6] failed to significantly inhibit VM using an endostatin analog. One possible explanation for this is that this type of therapy only targets angiogenesis but may not have any effect on VM because tumor cells lack the appropriate receptors for such inhibitors to act effectively [45]. Therefore, therapies that target both VM and angiogenesis would be required.

VM is often difficult to assess histologically and findings are often inconclusive [1]. Our study suggests the in vitro presence of VM in a small cohort of pituitary macroadenomas. We were unable to demonstrate a significant correlation between the presence of VM and tumor behaviour. We attribute this to a small sample size and insufficient data to draw definitive conclusions. A possible link between VM and age was also observed, although more studies are needed to confirm its existence.

Whether VM is present and clinically significant in pituitary adenomas is unknown. New methods are also required to identify VM in histological specimens of tumors. In this study, we provided evidence of the presence of VM in clinically non-functioning pituitary adenomas proposing the co-existence of different microvascular patterns (endothelium and non-endothelium based) in some subtypes of tumors.

In consideration of the need to find new biomarkers to facilitate early detection of clinically aggressive pituitary adenomas [46], VM should be investigated as a potential marker and/or therapeutic target. Further investigations are necessary as VM can have important implications for patients with respect to clinical course and therapeutic choices.

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Compliance with Ethical Standards

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Ethical Standards The authors wish to declare that they have no conflict of interest. All authors have contributed to, critically reviewed and approved this article. This work has been conducted with the consent of the patients and in accordance to the St. Michael's Hospital Ethics Committee where the work was undertaken. All work also conforms to the provisions of the Declaration of Helsinki.

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