Correlation of density of microvessels and myofibroblasts with the aggressiveness of central giant cell granulomas of jaws: A preliminary study

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Abstract

Objectives: Central giant cell granuloma (CGCG) is a fairly common lesion involving the jaw bones. CGCG can show relatively innocuous biological behaviour or it may show clinicoradiological features suggestive of aggressive biological behaviour. To date, there are no histological parameters which can be used to predict the behaviour of these lesions. This study was conducted to assess the utility of parameters of angiogenesis, i.e., total vascular area (TVA), mean vascular area (MVA) and microvessel density (MVD), and density of myofibroblasts in aggressive and non-aggressive CGCGs.

Materials and Methods: The study was undertaken as a retrospective study. A total of 20 previously diagnosed cases (10 non-aggressive and 10 aggressive) of CGCGs were included in the study. The sections were subjected to immunohistochemistry using the markers CD34 and α-SMA. For the assessment of vascular parameters, image J software was used. The density of myofibroblasts was determined in each case ranging from score-1 to 4, using the criteria given by Sridhara *et al*. The correlation between mean values of vascular parameters and density of myofibroblasts with aggressiveness of CGCG was assessed using Mann–Whitney U test.

Results: The result of Mann–Whitney U test suggested that the differences between the values of TVA ($P < 0.001$), MVA ($P < 0.003$) and density of myofibroblasts, i.e., SMA mean ($P < 0.001$) and SMA score ($P < 0.001$), in two groups are statistically significant. The formula for the assessment of aggressiveness was obtained using discriminant analysis.

Conclusions: Angiogenesis and density of myofibroblasts significantly differ in aggressive and non-aggressive cases of CGCGs. The aggressiveness of CGCG case can be predicted using the obtained formula by entering the values of vascular parameters and myofibroblasts.

Keywords: Aggressiveness, angiogenesis, central giant cell granuloma, microvessels, myofibroblasts

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INTRODUCTION

Central giant cell granuloma (CGCG) is a benign, proliferative, intraosseous lesion comprising 7% of all benign jaw lesions.[1] The term giant cell reparative granuloma was first used by Jaffe in 1953 to distinguish these lesions from giant cell tumour of long bones. He described it as a reactive lesion of the jaw bones occurring due to trauma, resulting in intraosseous haemorrhage and containing giant cells. Later the term GCRG was changed to CGCG, deleting the word reparative owing to the destructive rather than the reparative nature of CGCG.[2]

In the jaws, CGCG shows a benign but variable clinical behaviour and unpredictable course.[2,3] It is well accepted that prediction of the behaviour of these lesions using histological means is difficult.[4] However, Waldron and Whitaker produced a list of features that identified the cases that were significantly different between recurrent and non-recurrent and aggressive and non-aggressive ones. The non-aggressive cases usually show relatively innocuous clinical behaviour and mild symptoms. However, there is a subset of similar lesions which exhibit features suggestive of destructive biological behaviour such as pain, paraesthesia, root resorption, cortical perforation, etc. Such lesions are even prone to recurrence. Thus, they are designated as 'Aggressive' central giant cell lesions.[2] Several attempts to use immunohistochemical staining to study the role/function of the giant cells and the mononuclear cells in these lesions have been performed.[5-7] However, there are no established histological markers which can predict the biological behaviour of these lesions.

As a measure of angiogenic activity, most studies count the number of microvessels in tissue sections, which is expressed as mean vessel density (MVD). This technique was designated as an easy prognostic indicator for clinical behaviour for a number of tumours.[8,9] Many markers have been used to observe angiogenesis and factors influencing it in various lesions. CD34 is one of the sensitive immunohistochemical markers for vascular endothelium of both benign and neoplastic tissues as these molecules are found in association with endothelial microprocesses occurring at the tips of vascular sprouts suggesting that they play a role in cell adhesion and/or migration. It has been speculated that CD34 is produced by endothelial cells and associated with angiogenesis.[9]

Myofibroblasts (MF) are fibroblasts with smooth muscle-like features characterised by the presence of a contractile apparatus. Alpha-smooth muscle actin (α-SMA) is commonly regarded as the most important marker for myofibroblasts.[10] According to studies the mononuclear stromal cells, both histiocytes and myofibroblasts, have been thought to be responsible for the behaviour of the lesion like central giant cell granuloma.[11]

From the available literature, it seems that assessment of angiogenesis together with assessment of the density of myofibroblasts in central giant cell lesions may be helpful to predict their clinical behaviour. To date there are no studies, which show the assessment of angiogenesis and density of myofibroblasts together in central giant cell lesions, with a possible association to their biological behaviour. Thus, this study was conducted to evaluate the immunoexpression of CD -34 and $\alpha-$ SMA in CGCLs and to find out the correlation, if any, of immunoexpression of above said markers with the aggressiveness of CGCG.

MATERIAL AND METHODS

The study included a total of 20 previously diagnosed cases of CGCG of the jaws, from the archives of Department of Oral Pathology and Microbiology, Manubhai Patel Dental College, Vadodara. These cases were designated as aggressive or non-aggressive based on their clinicoradiological profiles at the time of diagnosis.

10 cases were of aggressive CGCG constituting group 1 and the other 10 cases were non-aggressive CGCG constituting group 2. Paraffin blocks of all cases were retrieved.

3 μm thickness sections were prepared from each block. At least one section was stained with haematoxylin and eosin stain [Figure 1]. Other sections were subjected to immunohistochemistry for CD34 and $\alpha-$SMA as follows:
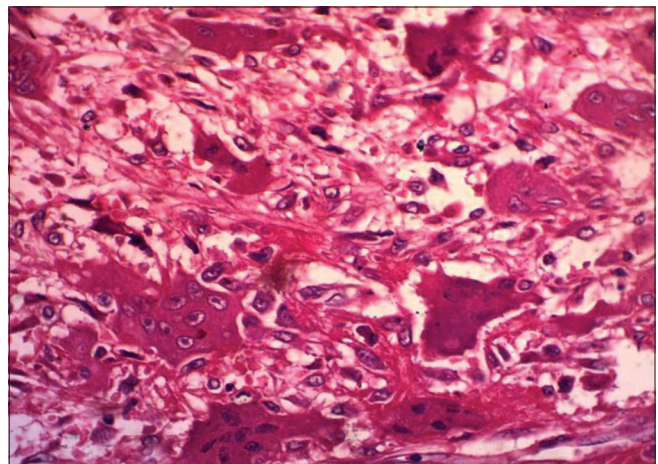

Figure 1: Photomicrograph showing microscopic features of central giant cell granuloma (Haematoxylin and Eosin stain; Magnification 400×)

Immunohistochemistry procedure for CD 34 and α – SMA

Sections were incubated at 37°C overnight before the day of staining, and then at 65°C for half an hour in morning on the day of staining followed by deparaffinisation, rehydration and antigen retrieval. All tissue sections were subjected to antigen retrieval using pressure cooker filled with citrate buffer solution (pH 6.0). Sections were incubated in hydrogen peroxide for 10 min to block endogenous peroxidase and washed in Tris buffered saline (TBS). Following this, the sections were treated with primary monoclonal antibody (CD 34 mouse monoclonal antibody, clone QBEnd 10, Dako North America, Inc. Carpinteria, CA, USA) for 45 min. Subsequently, the sections were incubated with Dako EnVision Horseradish peroxidase (HRP) labelled polymer for 30 min, washed in TBS before applying and also after applying HRP labelled polymer. Then, the sections were treated with DAB chromogen for 10 min, washed in distilled water and counterstained with Harris haematoxylin for 10 s. Finally, the sections were dehydrated, cleared and mounted with DPX.

The same procedure was repeated for immunohistochemical staining of myofibroblasts using anti-α – SMA antibody (Mouse monoclonal antibody, clone 14A, BioGenex, Fremont, CA, USA).

Immunoexpression of CD-34 and its morphometric analysis

Mean vascular density (MVD), total vascular area (TVA) and mean vascular area (MVA) were determined by evaluation of the expression of CD34 in each case with help of Image J software.

Endothelial cells showed membrane as well as cytoplasmic expression of CD 34 [Figure 2]. Photomicrographs were captured with the help of Lawrence and Mayo Research Microscope [LM52-1802, Aspire; Lawrence & Mayo (India) Pvt.Ltd] using TS view software at 400 × magnification. For morphometric analysis, photomicrographs of four representative hotspots of each case were subjected to computer-aided image analysis – ImageJ software (version 1.50i; Java 1.8.0_77). The morphometric analysis was performed manually as follows:

1. The chosen image file was dragged and dropped onto the Image J dialogue box [Figures 3 and 4].
2. The wand (tracing) tool was used and the tolerance was set to such a value so that an entire single blood vessel was traced [Figures 5 and 6].
3. Once the blood vessel was selected, the vascular area was obtained in a separate dialogue box of results for that single particular vessel by clicking on 'analyse' and then on 'measure' [Figure 7].
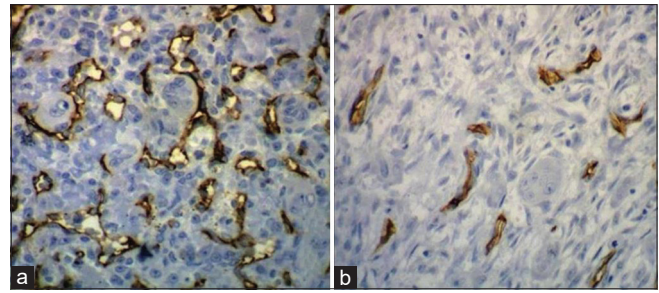

Figure 2: CD34 immunoexpression highlighting the blood vessels in CGCG (a: CD 34 in aggressive CGCG, b: CD 34 in non-aggressive CGCG) (Immunohistochemical stain; magnification 400×)
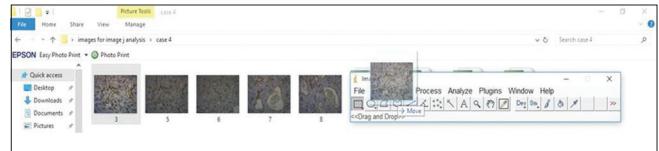

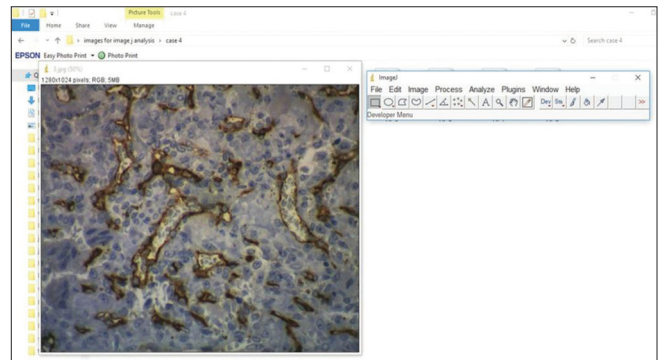**Figure 3:** Importing the image to Image J software by dragging and dropping

Figure 4: Opening the image in Image J software after dragging and dropping

4. After analyzing all the blood vessels in the particular hotspot, the dialogue box of results showed the vessel count contained in that particular hotspot along with their vascular area [Figure 8].
5. A similar procedure was repeated for all the remaining hotspots of the particular case.
6. Once the total number of blood vessels and vascular area were obtained for all the 4 hotspots for a particular case, the morphometric parameters – MVD, TVA and MVA – were calculated as the formulas given by Gadbail *et al*.[12]

Mean vascular density (MVD)

Total number of blood vessels in 4 high power fields or hotspots of a particular case/Total number of hotspots for that particular case, i.e., 4.

Total vascular area (TVA)

Total vascular area in 4 high power fields or hotspots of a particular case/Total number of hotspots for that particular case, i.e., 4.
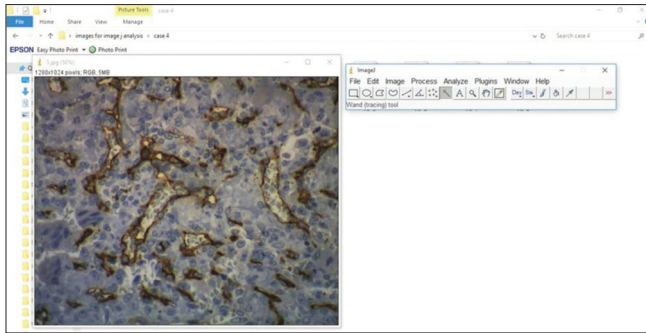
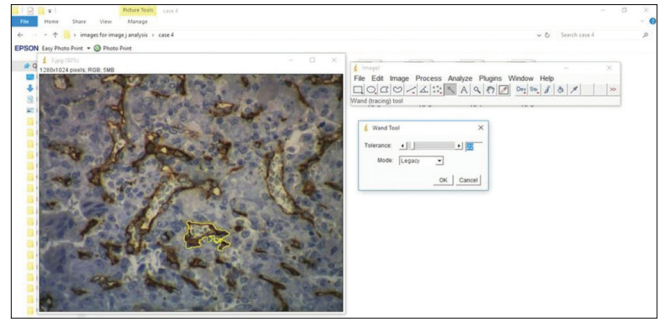**Figure 5:** Selection of the wand tool to trace the desired area in Image J

Figure 6: Tracing the perimeter of blood vessels
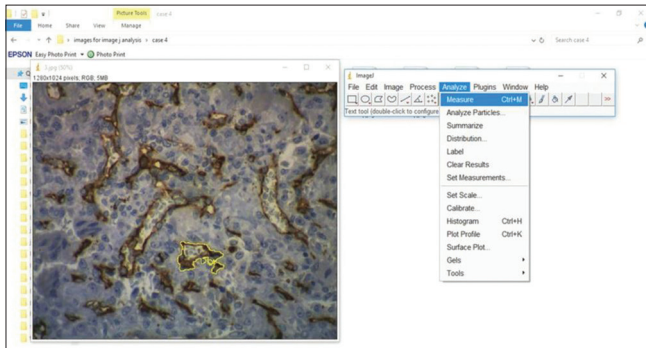

Figure 7: Selection of the tools, 'Analyse' followed by 'Measure' to obtain the area of the traced blood vessel
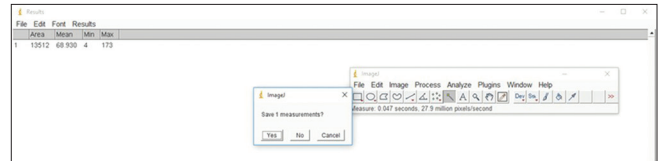

Figure 8: Vascular area obtained in the dialogue box

The TVA thus obtained was in pixels, and so it was converted into millimetres.

MVA was calculated as MVA = TVA/MVD

Analysis of immunoexpression of α-SMA

The density of myofibroblasts was determined by the evaluation of the expression of $\alpha - SMA$ in each case. For the quantitative analysis of α–SMA labelled myofibroblasts, 10 high power (Magnification: 400×) fields were randomly selected near the invasive front of the primary tumour and were examined under a light microscope.

The scoring of immunopositive cells was recorded quantitatively according to Sridhara *et al.*[13] [Figure 9]

Score 1 = no positive cells/<20 cells.

Score 2 = 21–100 positive cells.

Score 3 = 101–400 positive cells.

Score 4 = more than 400 positive cells.

The slide was moved in a zigzag manner (from right to left then left to right) to avoid repetition of the already examined field. The scores obtained were further calculated for mean positive cells per case and per study group.

The association between the aggressiveness of the lesion and density of myofibroblasts was further assessed by performing a Chi-square test. The result of the Chi-square test showed that the association between α-SMA score and aggressiveness of CGCG is statistically significant ($P < 0.001$).

The correlation between various parameters of angiogenesis, density of myofibroblasts and aggressiveness of the lesion was determined with the application of Mann–Whitney test. After obtaining the association between the parameters of angiogenesis, density of myofibroblasts and aggressiveness of CGCG cases, discriminant analysis was performed to set the formula to assess the aggressiveness of CGCG lesions.

RESULTS

Table 1 shows the mean values of MVD, TVA, MVA, SMA mean and SMA score in both groups. Mann–Whitney U test was carried out to find the significance of differences in mean values of vascular parameters between the two groups [Table 2]. Results of the test revealed that the differences between mean values of TVA ($P < 00.001$), MVA ($P < 0.003$), SMA Mean ($P < 0.001$) and SMA Score ($P < 0.001$) of two groups are statistically significant. However, the difference between mean values of MVD was not statistically significant ($P = 0.570$). These results suggest that TVA, MVA and number of α-SMA positive cells are significantly increased in the aggressive cases of CGCG [Table 3].

With the help of above-mentioned results, discriminant analysis was performed to set the formula to assess the aggressiveness of CGCG lesions [Tables 4 and 5]. The

Table 1: General data, vascular parameters and density of myofibroblasts in aggressive and non-aggressive cases of central giant cell granulomas

	Age	Gender	Site	MVD	TVA (mm²)	MVA (mm²)	α-SMA Mean	α-SMA Score
AGGRESSIVE	22	Female	Mandible	14.25	416.99	29.26	40.8	4
	32	Male	Mandible	12	395.81	32.98	31.4	3
	35	Male	Mandible	6	316.66	52.78	25.1	3
	27	Female	Maxilla	13.75	258.89	19.54	16.4	3
	25	Female	Mandible	7.25	297.74	41.06	56.7	4
	30	Female	Mandible	5.25	246.64	46.98	15.4	3
	31	Male	Mandible	6	212.72	35.45	22.4	3
	20	Female	Mandible	5.75	205.92	35.81	42.5	4
	36	Male	Maxilla	8.5	208.66	24.55	52.9	4
	19	Female	Mandible	6.25	197.26	31.56	39.5	3
NON-AGGRESSIVE	27	Female	Mandible	11	7.12	78.33	8.9	2
	22	Female	Mandible	9.75	85.93	8.81	8.1	2
	23	Male	Mandible	4.5	67.73	15.05	1.9	1
	30	Female	Mandible	9.75	116.97	11.99	2.7	2
	33	Male	Mandible	6.75	130.99	19.41	2.8	2
	31	Female	Maxilla	8.5	116.53	13.7	1.1	3
	18	Female	Mandible	10.75	97.81	9.09	1.7	1
	27	Male	Mandible	8.25	145.2	17.6	1.6	1
	24	Female	Mandible	10	73.53	7.35	1.3	1
	31	Female	Mandible	6.75	141.37	20.94	1.2	1

Table 2: Mann–Whitney U test for the assessment of the association between vascular parameters and density of myofibroblasts

	Group	N	Mean Rank	Sum of Ranks
MVD	Aggressive	10	9.75	97.50
	Non-Aggressive	10	11.25	112.50
	Total	20		
TVA	Aggressive	10	15.50	155.00
	Non-Aggressive	10	5.50	55.00
	Total	20		
MVA	Aggressive	10	14.40	144.00
	Non-Aggressive	10	6.60	66.00
	Total	20		
SMA Mean	Aggressive	10	15.50	155.00
	Non-Aggressive	10	5.50	55.00
	Total	20		
SMA Score	Aggressive	10	15.20	152.00
	Non-Aggressive	10	5.80	58.00
	Total	20		

Table 3: Result of Mann–Whitney U test

	MVD	TVA	MVA	SMA Mean	SMA Score
Mann–Whitney U	42.500	0	11.000	0	3.000
P	0.570	<0.001	0.003	<0.001	<0.001

Figure 9: Immunoexpression of α-SMA in CGCG cases (a: Score 1, b: Score 2, c: Score 3, d: Score 4) (Immunohistochemical stain; magnification 400×)

DISCUSSION

It is well established that vascularity and proliferative activity play key roles in tumour growth and invasiveness. Recently, it has been suggested that the degree of tumour angiogenesis is related to clinical outcome, suggesting that angiogenic properties correlate with tumour aggressiveness.[14]

In the present study, mean rank of MVD within the aggressive group was lower than that of non-aggressive lesions; however, statistical analysis showed no significant difference ($P = 0.57$). This result is in agreement with the previous study by O'Malley *et al.*,[15] who demonstrated no significant difference between aggressive and non-aggressive lesions by counting the percentage of CD 34 cells in these lesions.

following formula was found for the assessment of aggressiveness of the lesion.

Aggressiveness = -3.274 – 0.112(MVD) + 0.013(TVA) + 0.02(MVA) + 0.065 (SMA mean)

According to above-said formula, the value was near to the centroids (for non-aggressive cases near -2.334 and aggressive cases near 2.334.) After using the data of every single case in the discriminant analysis formula, 100% predictability was obtained for the assessment of the aggressiveness of the case. Overall predictive accuracy was also 100% according to the classification results.
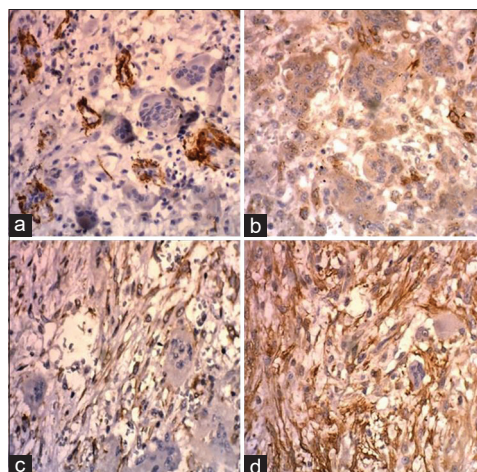
Table 4: Discriminant analysis for the aggressiveness of CGCG cases

Eigen value	% of Variance	Canonical Correlation	Box's M	*P*-value
6.053	100.0	0.926	67.804	<0.001

Table 5: Canonical discriminant functional coefficients

Variables	Function coefficients
MVD	−0.112
TVA	0.013
MVA	0.020
SMA Mean	0.065
(Constant)	−3.274

In the present study, the mean rank of TVA and MVA is higher in the aggressive group compared to the non-aggressive group and also statistically significant (*P* < 0.001 and *P* < 0.003 respectively). Thus, it can be considered that vascular parameters such as TVA and MVA can be useful to assess the aggressiveness of CGCGs.

Myofibroblasts (MFs) possess several distinguishing morphologic features and are characterised by the highly contractile α-SMA apparatus, which is also the most significant marker of myofibroblasts.[16] They actively participate in diseases characterised by tissue fibrosis because of their ability to secrete and degrade extracellular matrix components. Therefore, MFs are unique contractile cells that play a role in not only growth, development and wound healing but also in inflammation, fibrosis and tumour progression.[16] MFs are known to contribute to the biological behaviour of various lesions. Studies suggest an association between myofibroblasts (MFs) and the biological behaviour of odontogenic cysts and tumours.[10] The increased presence and the frequency of MFs in the stroma are directly related to more aggressive behaviour of such lesions.

According to the present study, the expression of α-SMA was increased in aggressive CGCG, which is suggested by the increased mean number of cells and score value in aggressive lesions. This result is in agreement with the previous studies.[17] Thus, myofibroblasts are important components of stromal cells in central giant cell lesions and alpha-smooth muscle actin immunoexpression was associated with features of local aggressiveness such as root resorption and cortical bone involvement.[15,18] Moreover, the pattern of distribution of these mononuclear myofibroblastic cells around the abnormal vessel spaces suggested that these cells might play a role in generating newly formed blood vessels and spaces.

Thus, it appears that vascular parameters TVA and MVA, and density of myofibroblasts are consistently different

in aggressive and non-aggressive lesions of CGCG. The vascular parameter MVD independently may not be associated with the aggressiveness of the lesion. However, the combined application of all three vascular parameters with mean number of myofibroblasts may become a promising method for the prediction of the biological behaviour of CGCGs. The current study was performed using the archival tissues of completely excised lesions. Future studies using the specimens of diagnostic biopsies of CGCGs are needed to explore whether the formula achieved through this research can guide the management of these lesions.

CONCLUSION

The results of the present study showed that compared to non-aggressive cases, aggressive CGCG cases show more density of myofibroblasts. Similarly, among various parameters of angiogenesis, values of TVA and MVA were higher in aggressive CGCG cases compared to non-aggressive cases. The density of myofibroblasts and angiogenesis may affect the biological behaviour of CGCG cases.

Parameters of angiogenesis and density of myofibroblasts can be used in combination to assess the aggressiveness of CGCG cases. The current study could not determine the age and gender predilection and site preferences for aggressive and non-aggressive CGCG cases. In future, studies with large sample size and post-treatment follow up may help to delineate such clinical differences between two groups. It can be recommended that parameters of angiogenesis and density of myofibroblasts, using CD 34 and α-SMA immunomarkers, should be investigated and used in combination in cases of CGCG to assess their aggressiveness.

Declaration of patient consent
The authors certify that they have obtained all appropriate patient consent forms. In the form, the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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