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ORIGINAL ARTICLE

Increased expression of pro-angiogenic factors and vascularization in thyroid hyperfunctioning adenomas with and without TSH receptor activating mutations

Marilena Celano · Marialuisa Sponziello · Giovanni Tallini · Valentina Maggisano · Rocco Bruno · Mariavittoria Dima · Enrico Di Oto · Adriano Redler · Cosimo Durante · Rosario Sacco · Sebastiano Filetti · Diego Russo

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Abstract Autonomously functioning thyroid nodules (AFTN) are known to receive an increased blood influx necessary to sustain their high rate of growth and hormone production. Here, we investigated the expression of hematic and lymphatic vases in a series of 20 AFTN compared with the contralateral non-tumor tissues of the same patients, and the transcript levels of proteins involved in the control of vascular proliferation, including the vascular endothelial growth factor (VEGF) and platelet-derived growth factors (PDGF) and their receptors and the endothelial nitric oxide synthase (eNOS). In parallel, the expression of the differentiation markers sodium/iodide symporter (NIS), thyroperoxidase (TPO), thyroglobulin (Tg), and TSH receptor (TSHR) was also investigated. The data were further analyzed comparing subgroups of tumors with or without mutations in the TSHR gene. Analysis by means of CD31 and D2-40 immunostaining showed in AFTN an increased number of hematic, but not lymphatic, vessels in parallel with an enhanced proliferation rate shown by increased Ki67 staining. Quantitative RT-PCR analysis revealed an increase of *VEGF*, *VEGFR*1 and 2, *PDGF-A*, *PDGF-B,* and *eNOS* expression in tumor versus normal tissues. Also, higher transcript levels of *NIS*, *TPO*, and *Tg* were detected. Comparison of the two subgroups of samples revealed only few differences in the expression of the genes examined. In conclusion, these data demonstrate an increased expression of angiogenesis-related factors associated with an enhanced proliferation of hematic, but not lymphatic, vessels in AFTNs. In this context, the presence of *TSHR* mutations may only slightly influence the expression of pro-angiogenic growth factors.

Keywords Thyroid toxic adenomas · TSH receptor mutations · VEGF · PDGF · eNOS

Marilena Celano and Marialuisa Sponziello contributed equally to this study.

M. Celano · V. Maggisano · D. Russo (✉)
Department of Health Sciences, University of Catanzaro "Magna Graecia", Viale Europa, Germaneto, 88100 Catanzaro, Italy
e-mail: d.russo@unicz.it

M. Sponziello · M. Dima · C. Durante · S. Filetti
Department of Internal Medicine and Medical Specialities, University of Roma "Sapienza", 00161 Rome, Italy

G. Tallini · E. Di Oto
Section of Anatomic Pathology, Bellaria Hospital, University of Bologna School of Medicine, 40139 Bologna, Italy

R. Bruno
Ospedale di Tinchi-Pisticci, 75100 Matera, Italy

A. Redler
Department of Surgical Sciences, University of Roma "Sapienza", 00161 Rome, Italy

R. Sacco
Department of Medical Sciences, University of Catanzaro "Magna Graecia", 88100 Catanzaro, Italy

Introduction

Autonomously functioning thyroid nodules (AFTN) are the second most frequent cause of hyperthyroidism and thus, they can have repercussions on the cardiovascular system and skeleton as well as on other organs and systems [1]. The presence of activating mutations of the TSH receptor (TSHR) gene has been reported in these tumors, when presenting as sporadic disease occurring either as single or in a multinodular goiter, in a sporadic or familial disease [2, 3].

Table 1 Somatic TSH receptor mutations in a subgroup of AFTNs

	Codon	Base substitution	Amino acid change
1	453	ATG → ACG	Methionine → isoleucine
2	568	ATC → TTC	Isoleucine → phenylalanine
3	632	ACC → ATC	Tyrosine → isoleucine
4	453	ATG → ACG	Methionine → isoleucine
5	486	ATC → ATG	Isoleucine → methionine
6	631	TTC → TTG	Phenylalanine → leucine
7	632	ACC → ATA	Threonine → isoleucine
8	633	GAC → TAC	Aspartic acid → tyrosine
9	403	Deleted GAT	Deleted aspartic acid

The functional activity of these mutations has been previously reported [34, 35]

More than 30 different amino acid substitutions have been described so far, and while their pathogenic role for the growth and function of the autonomous nodule is clearly established, the contribution of other factors to the acquisition of the full phenotype of the disease [3, 4] is still debated. Indeed, activating TSHR mutations have been also described in other thyroid diseases as hyperfunctioning carcinomas or "compensated" familial hypothyroidism [5, 6].

An increased blood supply provided by enhanced vascularization is a known feature of these tumors. Indeed, studies in small series of toxic adenomas have revealed changes in the secretion or expression of the vascular endothelial growth factor (VEGF) [7, 8] and platelet-derived growth factors (PDGF) [9], but the global gene expression profile of these factors and their receptors in these tumors has never been investigated. Moreover, there are no data regarding the influence of the TSH receptor mutations on the tumor vascularization and the expression of the angiogenic factors as well as their relationship with other markers of thyrocyte differentiation such as sodium iodide symporter (NIS), TSHR, Thyroglobulin (Tg), and Thyroperoxidase (TPO).

Herein, we analyzed the microvessel and lymphatic vessel densities in a series of autonomously functioning thyroid adenomas including tumors with and without activating mutations of the TSH receptor gene. We also investigated the gene expression of the following: the growth factors VEGF-A, PDGF-A and -B; the growth factor receptors VEGFRs 1, 2, 3 and PDGFR-α and -β; the endothelial nitric oxide synthase (eNOS); and the thyrocyte differentiation markers TSHR, NIS, Tg, and TPO.

Materials and methods

Tissue samples

All human tissue samples used in the study had been collected with full patients' consent and institutional review board approval. They included 19 AFTN and the non-tumor tissues from the contralateral lobe (as respective controls) of the same patients, living in an area of moderate iodine deficient supply [10], collected at Tinchi-Pisticci Hospital (Matera, Italy). The specimens have already been used in other studies of our group [11, 12] in which genetic examination had revealed the presence of somatic TSH receptor mutation in a subgroup of tumors (TSHR-mut AFTN, $n = 9$) (Table 1). Immediately after removal, part of each tissue specimen had been snap frozen in liquid nitrogen and stored at −80 °C for RNA isolation; the rest was fixed in Bouin's fixative overnight for histological studies. Before use, representative sections of the frozen tissue were cut and examined histologically to confirm the diagnosis and to avoid the contamination of a normal sample with nucleic acids from tumor-surrounding tissues.

RNA extraction, cDNA synthesis, and real time PCR

Gene expression analysis was determined by real time quantitative RT-PCR in a 7900HT Fast Real Time PCR System (Applied Biosystems, Foster City, CA, USA), as previously described with minor modifications [13]. Briefly, specific Assays-on-Demand gene expression products (Applied Biosystems) for each gene were selected to evaluate mRNA levels of angiogenesis-related genes (*VEGF-A*; *VEGFRs 1*, *2*, and *3*; *PDGF-A* and *-B*; *PDGFR-*α and *-*β; *eNOS*) and thyroid-specific markers of differentiations (*TSHR*; *NIS*; *Tg*; *TPO*) in each of the 19 AFTN samples and in a control group consisting of the corresponding contralateral non-tumor tissue of the patients. The β-actin gene was used as endogenous reference (Pre-Developed TaqMan Assay Reagents; P/N 4326315E; Applied Biosystems). Results, determined by the $2^{-\Delta\Delta Ct}$ method [14], were normalized to corresponding calibrator samples.

Analysis of microvessel and lymphatic vessel densities and cellular proliferation

Immunohistochemical studies included semiquantitative assessments of microvessel densities (blood and lymphatic)

and proliferation rate. They were performed according to standard procedures [15] on routinely processed, formalin-fixed sections from six of the 19 tumors, including the non-tumor counterparts, analyzed by RTQ-PCR. Microvessel density (MVD) was assessed with a commercial antibody (Cell Marque, Rocklin, CA, USA) that recognizes the CD31 marker expressed by endothelial cells of blood and lymphatic vessels. Microscopic assessment of lymphatic vessel density (LVD) was done with the Cell Marque's mouse monoclonal antibody clone D2-40, which recognizes podoplanin, a marker specific for lymphatic endothelial cells. Cellular proliferation was assessed with the anti-human Ki67 Antigen (Clone MIB-1) (DAKO, Glostrup, Denmark). Immunostaining for CD31, D2-40, and Ki67 was performed with a BenchMark XT automated platform (Ventana Medical Systems, Tucson, AZ) by means of a biotin-free horseradish peroxidase polymer detection method (Ultravision LP, LabVision Corporation, Fremont, CA, USA). Labeling in tumors and adjacent non-neoplastic thyroid tissue was rated as follows. The Weidner protocol, with minor modifications, was used to determine MVD and LVD [15]. Individual vessels were counted at high power (200×), and the averages of the counted vessels were scored as 1(<30 vessels), 2 (30–60 vessels), 3 (>60 vessels) for CD31, as 1(<5 vessels) and 2 (5–20 vessels), 3 (>20 vessels) for D2-40. The Ki67 labeling index (i.e., the percentage of cells with positive Ki67 nuclear immunoreactivity) was used to determine cellular proliferation, following previously published protocols [16].

Statistical analysis

Quantitative RT-PCR results are expressed as mean ± SD, and the significance of differences was assessed by the Mann–Whitney nonparametric test, P values <0.05 were regarded as statistically significant. Data analysis was performed by means of StatView 5.0.1 software (SAS Institute Inc., Cary, NC, USA).

Results

Immunohistochemical analysis of pro-angiogenic protein expression and vessel densities

Six samples representative of the two subgroups of tumors (TSHR-mut and TSHR-wt AFTNs) were analyzed by immunohistochemistry using CD-31, D2-40, and Ki67 as markers of vessel density and proliferation rate. Results are summarized in Table 2 and illustrated in Fig. 1. As expected, Ki67 staining demonstrated proliferating neoplastic cells, with variable labeling indexes (range 2–6 %), but always higher than the corresponding non-tumor one, in the samples analyzed for gene expression analysis (see below). All AFTNs exhibited numerous CD31-positive intra-tumor microvessels (Fig. 1) with consistently higher MVD scores for toxic adenomas compared with controls. D2-40 immunostaining demonstrated the network of lymphatic vessels in the non-neoplastic thyroid parenchyma. However, inside the adenoma, lymphatic vessels were virtually absent with markedly reduced LVD scores compared with controls.

No significant differences appeared between specimens belonging to each subgroup of mutated or non-mutated samples for all the markers examined (data not shown).

Gene expression analysis

A series of 19 AFTN tissues were analyzed for the expression level of major pro-angiogenic factors by real time quantitative PCR. Compared with the non-tumor tissues, the AFTNs displayed significantly higher mean mRNA levels for all the growth factors *VEGF-A*, *PDGF-A* and *PDGF-B*, the receptor *VEGFR1*, *2*, and the *eNOS* and not significantly different mean levels of *VEGFR3* and *PDGFR-α* and *-β* (Fig. 2a). Subgroup analysis of the AFTNs showed that transcription of *PDGFR-β* and *eNOS* was significantly higher in the TSHR-mut tumors (Fig. 2b).

Analysis of the expression of the thyrocyte differentiation markers showed a significant increase in the mRNA levels of *NIS*, *TPO,* and *Tg*, but not the *TSHR* in the AFTN compared with their normal counterpart (Fig. 3a). TSHR-mut subgroup showed *TPO* mRNA levels significantly higher than TSHR-wt subgroup (Fig. 3b).

Discussion

Vascularization is fundamental for supporting tumor cell growth rate and, in case of malignancy, also metastatic diffusion. Thus, the mechanisms underlying this process have been the object of investigation for all epithelial tumors including thyroid ones. Many studies have reported the presence of endothelial cell proliferation in thyroid tumors being associated to increased expression of pro-angiogenic factors; therefore, identification of the profile of pro-angiogenic factors expressed and their relationship with other tumor biomarkers may help to provide novel and additional therapeutic targets. Indeed, many preclinical studies have been performed and clinical trials are in progress using novel small molecules provided with anti-angiogenic activity [17–19].

While many data are available regarding such an important issue in thyroid malignancies, only few studies have investigated the mechanism responsible for the increased vascularization occurring in AFTNs and their

Table 2 Immunohistochemical analysis of vessel densities and proliferation rate

Case		CD31	D2-40	Ki67
2	T	3	1	2
	N	1	2	<1
9	T	2	1	5
	N	1	2	<1
11	T	2	1	4
	N	1	3	<1
12	T	3	1	6
	N	1	3	<1
15	T	2	1	3
	N	1	2	<1
16	T	3	1	2
	N	1	2	<1

The average vessel counts scored at 200× magnification are rated as follows: 1(<30 vessels), 2 (30–60 vessels), 3 (>60 vessels) for CD31; 1(<5 vessels), 2 (5–20 vessels), 3 (>20 vessels) for D2-40. The Ki67 labeling index is the percentage of cells with positive Ki67 nuclear immunoreactivity. Cases 2 and 9 belong to TSHR-mut subgroup. Cases 11, 12, 15, 16 belong to TSHR-wt subgroup

T tumor, *N* non-neoplastic tissue

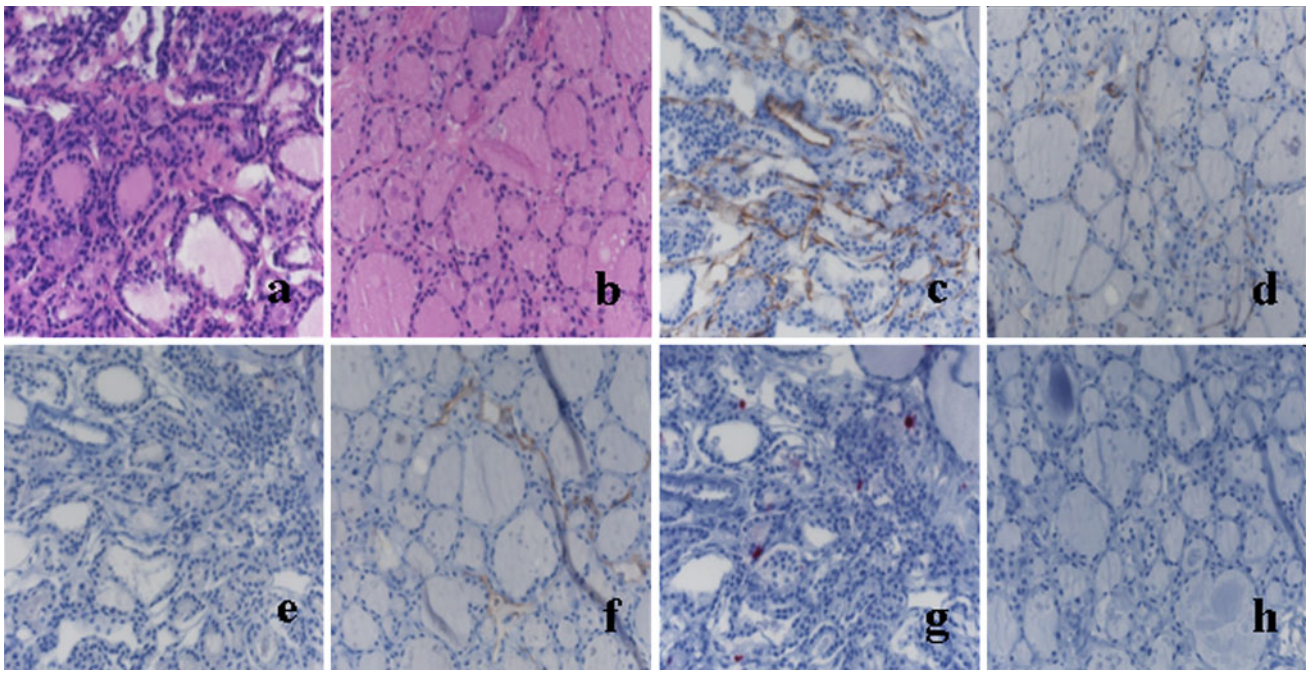

Fig. 1 Vessel density and proliferation in AFTN and corresponding normal control. Hematoxylin and eosin section of a representative case of AFTN (**a**) and of surrounding non-tumor tissue (**b**) (×200); the endothelial cells of numerous small-sized blood vessels stained *brown* after immunohistochemistry for CD31 (**c**); the corresponding normal thyroid parenchyma shows fewer capillary vessels than the tumor (**d**); *brown* D2-40 immunostain for lymphatic endothelial cells is virtually absent in the tumor (**e**), but present in the non-tumor tissue (**f**); *red* nuclear immunoreactivity for the proliferation marker Ki67 is present in scattered neoplastic cells (**g**), but absent in the surrounding non-tumor tissue (**h**)

relationship with the pathogenic alterations detected in these tumors. Our present data demonstrate the presence of an increased expression of the pro-angiogenic factors PDGFs and VEGF with their receptor 1 and 2, associated with an enhanced proliferation of hematic, but not lymphatic, vessels. An upregulation of VEGF-A is a well-known characteristic of thyroid proliferative lesions including benign and malignant lesions and Graves'

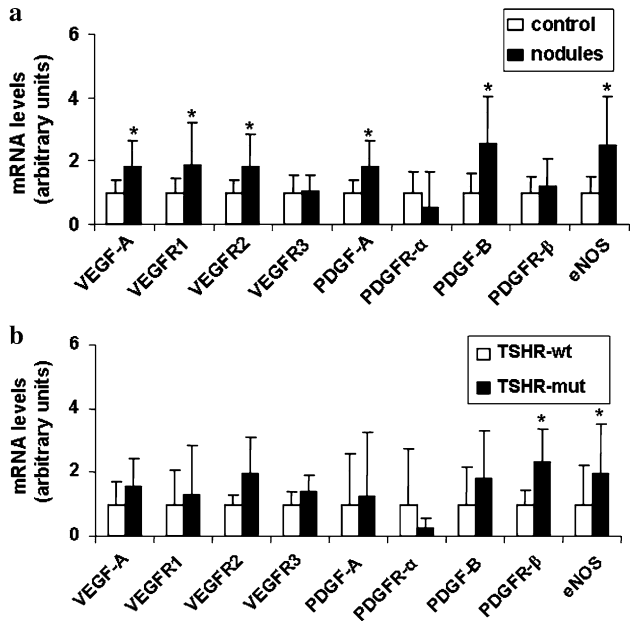

Fig. 2 mRNA levels of genes encoding pro-angiogenic factors. *VEGF-A*, *VEGFRs* 1, 2, and 3, *PDGF-A* and *-B*, *PDGFR-α* and *-β*, *eNOS* mRNA levels were measured in 20 AFTN samples and in a control group consisting of the corresponding contralateral non-tumor tissue of each patient (considered as 1) (**a**) and in TSHR-mut versus TSHR-wt subgroup (considered as 1) (**b**). Total RNA was extracted as described in the "Methods" section. *β*-actin was amplified as an endogenous control. Results are expressed as the mean ± SD. Intergroup differences were assessed by the Mann–Whitney nonparametric test. $*P < 0.05$

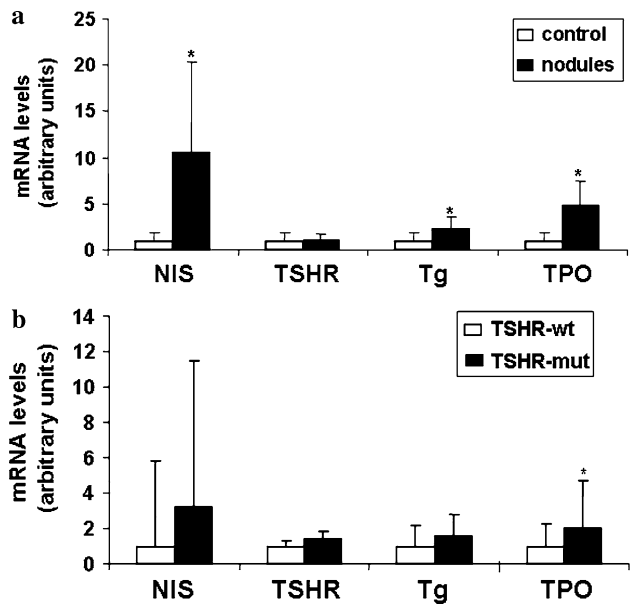

Fig. 3 mRNA levels of genes encoding thyroid differentiation markers. *NIS, TSHR, TG*, and *TPO* mRNA levels were measured in 20 AFTN samples and in a control group consisting of the corresponding contralateral non-tumor tissue of the patients (considered as 1) (**a**) and in TSHR-mut versus TSHR-wt subgroup (**b**). All values are mean ± SD. Intergroup differences were assessed by the Mann–Whitney nonparametric test. $*P < 0.05$

disease [7, 20, 21]. Also, expression of VEGF-A has been shown in vitro to be regulated using TSH, via cAMP and PKC pathways, and Graves' serum [22, 23]. Our study, which for the first time compared the mRNA levels of the AFTN with the corresponding normal tissues, confirms a role for VEGF to promote neovascularization as support for the tissue growth and function. Accordingly, and concordant with the results of Deleu et al. [24], increased Ki-67 staining (marker of cell proliferation) paralleled that of CD-34 used for evaluation of MVD. Also, in accordance with the data of Viglietto et al. [20] on Graves' tissues, expression of VEGF receptors 1 and 2 genes resulted increased in our series of tissues. Thus, the VEGF-related pathway appears as a major effector for the vascularization of toxic adenomas as in thyroid cancer. Moreover, for the first time, our data showed increased mRNA levels of both the isoforms of PDGF in the tumors versus the normal tissues, not correlated with those of the correspondent receptors. The contribution of such an additional pathway, previously demonstrated in thyroid cancer [15], remains to be clarified. Intra-tumor lymphatics development has been shown to occur in papillary thyroid cancer and is associated to the presence of nodal metastases [7, 25]. This feature was absent in our series of benign tumors, appearing therefore as a clear marker of malignancy. At variance with our present finding and those of Deleu et al. [24], Viacava et al. [8] found very low proliferative activity in 15 functioning adenomas compared with adjacent tissue. The well-known heterogeneity in the clinicobiological behavior of the AFTNs may, in part, account for such a discrepancy, especially for a marker expressed at very low levels in proliferating thyrocytes [16].

A relationship between eNOS expression and vascular control during goiter formation and hyperthyroidism has been already reported, including in tissues obtained from seven patients with Graves' disease and one with severe hyperthyroidism due to a germline mutation of the TSH receptor [26, 27]. Our present data obtained in a larger series of patients confirm such finding, proposing a role for NO as a vascular relaxing factor in proliferative and hyperfunctioning tissues. However, the activation of the NO pathway in concert with angiogenic factors has been described also in papillary thyroid carcinomas [28].

Investigation of the expression of the thyrocyte differentiation markers showed an expected increase in the mRNA levels of *NIS*, *TPO*, and *Tg*, consistent with the well-differentiated phenotype of these tumors and their increased rate of hormone synthesis. Also, the finding of a lack of significant difference in the TSHR gene expression between tumor and normal tissues was in some way expected since its constitutive activity (in the mutated samples) or unknown alterations presumably acting with a similar molecular mechanism (in the non-mutated samples)

make an increase of its expression levels not necessary to get a stimulation of both growth and function of the tumor.

A comparison between AFTN with and without TSHR mutation revealed very few significant differences in the expression of genes involved in angiogenesis (PDGFR-β and eNOS) or differentiation (TPO). A proposed explanation may relate to the above-mentioned unknown alterations present in the TSHR-wt subgroup, which likely involve the same signal transduction pathways (cAMP or Ca-IP dependent) constitutively activated in the mutated group. Alternatively, activation of "internal feedback loops" in the TSHR-mut subgroup, involving adenylate cyclase, phosphodiesterase, or functional CREB expression, may counteract the effects of the mutations [29–31]. Finally, a role for environmental factors in addition to the genetic alterations may largely influence the gene expression profile as it acts on the full phenotype of the disease, as previously documented [32].

Recently, novel drugs provided with anti-angiogenic effects, mediated in large part by blocking the activation of receptors for VEGF and PDGFs expressed on endothelial cells, were under investigation against thyroid malignancy [33]. Thus, selection of AFTNs expressing high levels of these proteins may be potential targets of such novel treatments.

In conclusion, these data demonstrate that increased expression of vascular growth factors is associated with an enhanced proliferation of hematic, but not lymphatic, vessels. In this context, the presence of TSH receptor mutations may only slightly influence the expression of growth factors only at transcription levels.

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Conflicts of interest The authors declare that the experiments comply with the current laws of the country in which they were performed and that they have no conflict of interest.

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