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ORIGINAL ARTICLE

Dysregulation in IGF-1R, FGFR4 and βKlotho signaling in patients with medullary thyroid cancer

Ewelina Motylewska[1*], Magdalena Borkowska[2*], Hanna Ławnicka[1], Krzysztof Kuzdak[2], Agnieszka Siejka[3], Jacek Świętosławski[4], Henryk Stępień[1], Tomasz Stępień[2]

1 Department of Immunoendocrinology, Chair of Endocrinology, Medical University of Lodz, Pomorska 251, 92-213 Lodz, Poland, tel/fax: +48 42 2014417
2 Clinic of Endocrinological and General Surgery, Chair of Endocrinology, Medical University of Lodz, Pabianicka 62, 93-513 Lodz, Poland, tel/fax: +48 42 6895171
3 Clinic of Endocrinology, Chair of Endocrinology, Medical University of Lodz, Pomorska 251, 92-213 Lodz, Poland. tel/fax: +48 42 2014420
4 Department of Neuroendocrinology, Medical University of Lodz, Pomorska 251, 92-213 Lodz, Poland.

*E. Motylewska and M. Borkowska contributed equally to this work

Correspondence to: Hanna Ławnicka MD, PhD
Department of Immunoendocrinology, Chair of Endocrinology, Medical University of Lodz, Pomorska 251, 92-213 Lodz, Poland
TEL./FAX: +48 42 2014417; E-MAIL: hanna.lawnicka@umed.lodz.pl

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Abstract

BACKGROUND: Medullary thyroid cancer (MTC) is a relatively rare thyroid neoplasm derived from neuroendocrine C cells which secrete calcitonin. αKlotho (αKL) and βKlotho (βKL) are transmembrane proteins which modulate different signaling systems, such as endocrine FGFs and IGF1 pathways. Dysregulation of the FGF19/FGFR4/βKL and IGF-1/IGF-1R/αKL signaling axes has been implicated in the pathogenesis of several cancers. However, their role in the pathogenesis of MTC has not been determined.
METHODS: The aim of this study was to assess αKL, βKL, FGF19, IGF-1, FGFR4, and IGF-1R concentrations in a group of 11 patients with medullary thyroid cancer (MTC). The control group consisted of 20 healthy volunteers. Serum concentrations of these factors were measured using specific ELISA methods.
RESULTS: Significantly lower concentrations of βKL and higher concentrations of FGFR4 and IGF-1R were found in patients with MTC as compared to controls.
CONCLUSIONS: Our results indicate that a disrupted signaling pathway for βKL, FGFR4 and IGF-1R may play a role in the development of medullary thyroid cancers. However, further studies are required to confirm these findings and to use this knowledge in clinical practice.

Abbreaviations:

ADAM	- a disintegrin and metalloproteinase;
AKT	- protein kinase B;
DTC	- differentiated thyroid cancer;
FGF	- fibroblast growth factor;
FGFR	- fibroblast growth factor receptor;
FTC	- follicular thyroid cancer;
GSK-3β	- glycogen synthase kinase 3;
HCC	- hepatocellular carcinoma;
IGF1	- insulin growth factor;
IGF-R	- insulin growth factor receptor;
JAK	- Janus kinase;
MAPK	- mitogen-activated protein kinases;
MTC	- medullary thyroid cancer;

PI3K - phosphatidylinositide 3-kinases;
PKC - proein kinase C;
PTC - papillary thyroid cancer;
sKL - secreted Klotho;
STAT - Signal Transducer and Activator of Transcription proteins;
TGF-β1 - transforming growth factor-β1;
αKL - αKlotho;
βKL - βKlotho

INTRODUCTION

Medullary thyroid cancer (MTC) is a relatively uncommon neuroendocrine neoplasm of thyroid C cells. Its origin and biology is different from the most frequent differentiated thyroid cancers (DTC) (papillary and follicular thyroid cancers). In 25% of MTC cases, the disease is hereditary, occurring as part of the MEN 2 syndrome due to germline activating mutations in the *RET* protooncogene. *RET* protooncogene encodes a single-pass transmembrane protein that belongs to the receptor tyrosine kinase family. In physiological conditions, once recruited, the RET protein will lead to the activation of multiple signaling pathways including JAK/STAT, PKC, PI3K/AKT, and RAS/MAPK. Several known mutations can convert the RET receptor into a dominant transforming oncogene. RET is also mutated in about 50% of sporadic cases of MTC. Moreover, in sporadic MTC, somatic mutations of RAS were also identified. In both hereditary and sporadic cases, specific *RET* mutations are correlated with phenotype and prognosis. MTC is relatively insensitive to chemotherapy. Therefore, targeted therapy is particularly needed. In recent years, tyrosine kinase inhibitors have provided significant clinical benefits in MTC treatment. However, better understanding of the C cell biology and its oncogenic transformation to MTC could be critical for achieving other effective targeted therapy (Raue 2015).

Klotho is a protein in which dysregulation was identified in several cancers (Zhou & Wang 2015). Klotho (αKL) was originally identified as an anti-aging gene (Kuro-o *et al.* 1997). It is expressed predominantly in renal distal convoluted tubules and brain choroid plexus and, to a lesser extent, in areas such as the parathyroid gland, thyroid gland, pancreas, and sex organs (Dalton *et al.* 2017; Kuro-o 2012). It encodes a type I single pass transmembrane protein composed of intracellular, transmembrane and extracellular domains with two internal repeats (KL1 and KL2). The extracellular domain can be cleaved by metalloproteinases ADAM10 and ADAM17, forming soluble Klotho of about 130 kDa, which is then released into the blood, urine or cerebrospinal fluid, where it acts as circulating hormone (secreted Klotho, sKL) (Dalton *et al.* 2017; Kuro-o 2012). Moreover, some studies have indicated that there is a second recognition site for these proteases located between the KL1 and KL2 domains, which generates two new 70-kDa isoforms, one containing the KL1 domain only and the other one containing the KL2 domain (Chen *et al.* 2014). Hence, there are two forms of Klotho protein: membrane and sKL. A secreted, truncated form that is produced by alternative splicing of klotho mRNA and consists of KL1 only was also reported (Dalton *et al.* 2017). Membrane Klotho protein functions as an obligate coreceptor for FGF23 (fibroblast growth factor), while sKL functions as humoral factor regulating the activity of oxidative stress, ion channels, ion transporters and multiple growth factor receptors on the cell surface (Kuro-o 2012; Yamamoto *et al.* 2005).

The *βKlotho* gene was identified based on sequence similarity with the *klotho* gene and shares 41.2 % amino acid identity with Klotho. It also encodes the single pass transmembrane protein (Ito *et al.* 2000). However, its tissue distribution differs from that of Klotho. βKL is expressed mainly in the liver, pancreas and in adipose tissue. It modulates FGF19 and FGF21 signaling as a cofactor for FGFRs (fibroblast growth factor receptor) (Kuro-o 2012). Presumably, βKL may be able to function in a similar manner to αKL as the cell-associated and the secreted extracellular portion of KL. This mechanism could facilitate FGFs to act in tissues where βKL is not normally expressed (Lin & Desnoyers 2012).

Thus, αKL and βKL modulate signaling of endocrine FGFs (comprising FGF19, FGF21 and FGF23) acting as coreceptors for their specific FGFRs (Itoh *et al.* 2015). The FGF19/FGFR4/βKL signaling axis is of particular interest in oncology as its deregulation at the ligand or receptor levels has been implicated in the pathogenesis of several cancers (Zhou & Wang 2015). Moreover, studies by re-expression of klotho in cancer cells have revealed that sKL acts as a tumor suppressor by inhibiting multiple other signaling pathways that include the insulin/IGF-1 (insulin-like growth factor 1) pathway, Wnt signaling pathway and TGF-β1 (transforming growth factor-β1) pathway (Dalton *et al.* 2017; Zhou & Wang 2015).

The aim of the present paper was to explore the role of Klotho proteins and some of their signaling systems in medullary thyroid cancer. IGF19/FGFR4/βKL and IGF-1/IGF-1R/αKL pathways were assessed in this study. Thus, serum concentrations of αKL, βKL, IGF19, IGF-1, FGFR4 and IGF-1R were determined in patients diagnosed with MTC and compared with healthy controls.

MATERIAL AND METHODS

Study Design and Patient Characteristics

Patients aged 18 to 75 years (49,60 ±7,82) (mean ± standard error of the mean) treated by surgery in the Clinic of Endocrinological and General Surgery, Copernicus Memorial Hospital, Lodz, Poland between 2012-2015 were enrolled to the study. The examined group was composed of 11 subjects diagnosed with MTC.

Selected cases were diagnosed by fine-needle biopsy and confirmed by postoperative histopathologic examination. Other thyroid gland pathologies were excluded

Tab. 1. Demographic characterization of the studied group (medullary thyroid cancer (MTC)) and healthy controls

Group	Number of patients	Gender F/M	Age (years) Mean ±SEM
Control	20	10/10	55.15 ± 9.80
MTC	11	7/4	49,60 ± 7,82

Tab. 2. Histopathological diagnosis and clinical staging of medullary thyroid cancer patients (MTC) included in the study, recommended by the 2010 TNM system edition by the Union for International Cancer Control

Diagnosis (n=number of patients)	Stage			
	I	II	III	IVA
MTC (n=11)	1	4	4	2

on the basis of familial and clinical history, clinical examination, ultrasonography and thyroid function tests (aTPO, aTG TSH, fT3, fT4). The control group included 20 healthy, age-matched volunteers with no history of any thyroid disease, confirmed by clinical, hormonal, thyroid ultrasound scan, and the absence of thyroid autoantibodies. Demographic and clinical characteristics of the examined group and healthy controls are presented in Table 1. All patients diagnosed with MTC were treated with total thyroidectomy, and therapeutic neck dissection was performed with standard indications. The histopathological diagnosis and clinical staging of thyroid cancer patients included in the study is presented in Table 2.

The project was approved by the Bioethics Committee of the Medical University of Lodz. Informed consent was obtained from all individual participants included in the study.

Measurements of βKlotho, FGF19 and FGFR4 Serum Levels by ELISA

Blood samples were collected from the antecubital vein between 7:00 and 8:00 am after an overnight fast, one day before surgery. Blood samples were processed within one hour after collection, and serum was aliquoted and stored at -80^{0}C until analysis. Serum concentrations of FGF19 (R&D Systems), αKlotho (IBL International GmbH), IGF-1 (Mediagnost GmbH) FGFR4, IGF-1R and βKlotho (Shanghai Sunred Biological Technology Ltd.) were evaluated using enzyme-linked immunosorbent assay (ELISA) kits, following the manufacturer's instructions. All measurements were taken in duplicate and averaged.

Statistical analysis

The results were presented as mean ± standard deviation (SD). The Shapiro-Wilk test was applied to analyze the data distribution. ANOVA followed by Fisher's protected Least Significant Difference was used to calculate differences between investigated groups; $p < 0.05$ was considered significant. The independent relationship between serum concentration of all examined factors was examined using Pearson's linear correlation analysis. All statistical analyses were performed using StatSoft statistical software v.12.0. (Statistica PL).

RESULTS

The quantitative determination of FGFR4, IGF-1R and βKL concentrations in the serum and the statistical evaluation of these results are presented in Fig. 1-3. The mean serum level of IGF-1R in MTC patients (35.6±9.4 ng/mL) was significantly higher than that obtained in controls (16.7±3.0 ng/mL) (Fig. 1). Similarly, the mean serum level of FGFR4 in MTC patients (33.0±7.3 ng/mL) was also higher than in controls (13.8±3.7 ng/mL) (Fig. 2). However, the mean serum level of βKL in MTC patients (4022.8±933.9 ng/L) was significantly lower than that observed in controls (7454.8±602.1 ng/L) (Fig. 3). The FGF19, IGF-1 and αKL levels in patients diagnosed with MTC did not differ significantly from those of healthy controls ($p>0.05$).

DISCUSSION

αKlotho is considered as universal tumor suppressor (Rubinek & Wolf 2016). Studies show that αKlotho is silenced by epigenetic mechanisms (promoter hypermethylation, histone deacetylation) and various microRNAs in a wide array of malignancies, including breast, pancreatic, lung, colorectal, gastric, cervical cancers and melanoma, and that Klotho expression can serve as an invaluable prognostic marker. In addition, accumulated data suggests that forced expression of Klotho or treatment with soluble Klotho can inhibit development and progression of different cancers (Rubinek & Wolf 2016; Zhou & Wang 2015), including, among others, follicular thyroid cancer (Dai *et al.* 2016). The tumor suppressive action is to a great extent connected to sKL which was revealed to inhibit multiple signaling pathways such as the insulin/IGF-1 pathway, Wnt signaling pathway and TGF-β1 pathway (Dalton *et al.* 2017; Zhou & Wang 2015). Therefore, in our paper, we determined the serum concentration of soluble αKL

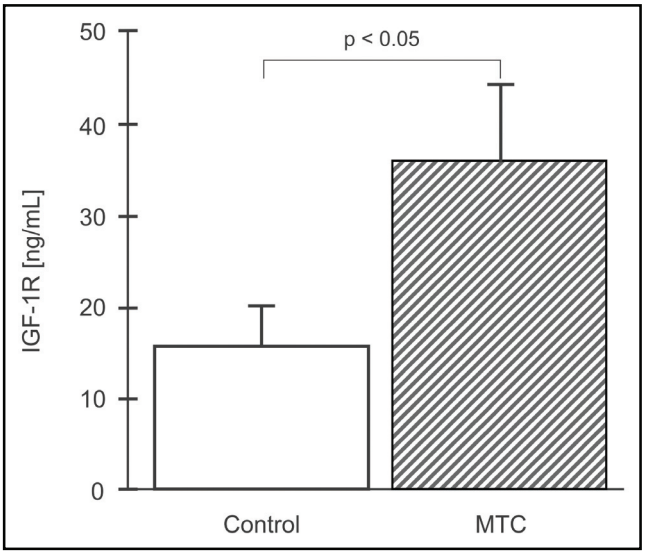

Fig. 1. IGF-1R in patients with medullary thyroid cancer. Mean serum concentration of IGF-1R (ng/mL) in patients diagnosed with medullary thyroid cancer (MTC) and in healthy volunteers (Control). Bars represent mean ± SD.

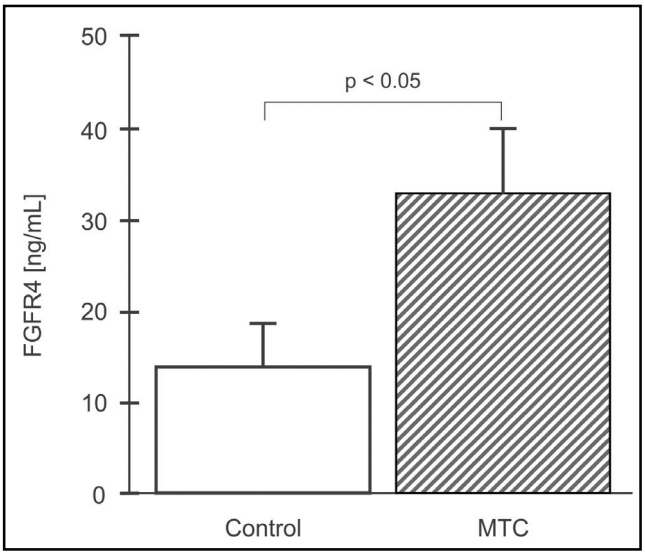

Fig. 2. FGFR4 in patients with medullary thyroid cancer. Mean serum concentration of FGFR4 (ng/mL) in patients diagnosed with medullary thyroid cancer (MTC) and in healthy volunteers (Control). Bars represent mean ± SD.

in MTC patients. However, no statistically important difference was observed in αKL levels between MTC patients and controls. No other studies assessed αKL in this tumor. Not much more is known about IGF-1 axis in pathogenesis of MTC, although its role in different neoplasms is well established. Insulin and IGF-1 bind to their receptors, activating IRS proteins and leading to activation of PI3K/Akt or MAPK/ERK1/2 cell signaling pathways, which play an important role in cell proliferation and apoptosis. Dysregulation of these pathways can lead to tumor development and progression (Pollak 2008). These intracellular signals of insulin and IGF1 could be repressed by sKL (Kurosu *et al.* 2005). Indeed, α-Klotho was revealed to act as

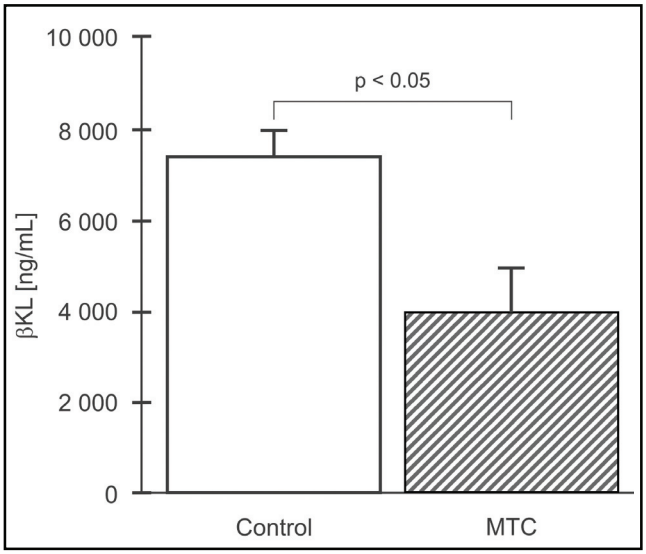

Fig. 3. βKL in patients with medullary thyroid cancer. Mean serum concentration of βKL (ng/L) in patients diagnosed with medullary thyroid cancer (MTC) and in healthy volunteers (Control). Bars represent mean ± SD.

a tumor suppressor by inhibiting insulin/IGF-1 signaling in breast cancer (Wolf *et al.* 2008; Ligumsky *et al.* 2015), lung cancer (Chen *et al.* 2010), pancreatic cancer (Abramovitz *et al.* 2011), gastric cancer (Xie *et al.* 2013), liver cancer (Shu *et al.* 2013) and colon cancer (Li *et al.* 2014). Overexpression of α-Klotho or treatment with sKL inhibits phosphorylation of IGF-1R and suppress insulin/IGF-1-mediated downstream effectors IRS-1, Akt1, and ERK1/2 in cancer cells (Abramovitz *et al.* 2011; Chen *et al.* 2010; Li *et al.* 2014; Ligumsky *et al.* 2015; Shu *et al.* 2013; Wolf *et al.* 2008; Xie *et al.* 2013). This tumor suppressive activity has been attributed to its KL1 domain (Abramovitz *et al.* 2011; Ligumsky *et al.* 2015). More importantly, in vivo administration of KL1 in mice showed that it may be as effective as the full-length protein for the treatment of cancer, but it may have a better safety profile (Abramovitz *et al.* 2011; Ligumsky *et al.* 2015).

The role of IGF system in differentiated thyroid cancers is quite well explored (Vella *et al.* 2001). In particular, insulin resistance is associated with increased thyroid volume (Rezzonico *et al.* 2008) and increased risk of thyroid nodules (Rezzonico *et al.* 2008) and DTC (Rezzónico *et al.* 2009). These findings have led to the hypothesis that the rising thyroid cancer incidence observed worldwide might be related to the rising occurrence of insulin resistance (Gursoy A 2010). Moreover, compared with nodular goiters, DTC showed significantly higher IGF-1R expression (Karaca *et al.* 2011; Liu *et al.* 2013). However, the IGF1 signaling system in MTC has been rarely explored so far. Thus, our study revealed for the first time that IGF-1R levels are also elevated in MTC patients. This is important information, as the increased signaling in this pathway could be potentially blocked by KL1 therapy or by other IGF-1R inhibitors. Interestingly, our study has

not revealed any difference in IGF-1 serum concentrations between the MTC group and healthy controls. Similarly, no difference in IGF-1 serum concentration was detected in MTC patients in comparison with different histologic types of thyroid carcinoma by other authors (Pazaitou-Panayiotou *et al.* 2016). Moreover, IGF1 expression was also comparable in MTC and in other thyroid neoplasms or normal thyroid tissues (van der Laan *et al.* 1995).

Less is known about the role of βKL in cancer development. However, βKL was demonstrated to be expressed in the epithelial cells of some prostate cancer tissues and cell lines with no expression in normal prostate epithelium (Feng *et al.* 2013). Similarly, other authors have identified an elevation of βKL gene expression in hepatocellular carcinoma (HCC) tumors relative to matched non-tumor tissue and reported that βKL-silencing in HCC cells decreased cell proliferation and suppressed FGFR4 downstream signaling *in vitro* (Poh *et al.* 2012). In contrast, other studies have found βKL expression to be down-regulated in HCC tissues compared with adjacent non-tumorous tissues. βKlotho overexpression also induced an anti-proliferative effect in hepatoma cells *in vitro* and inhibited tumorigenesis *in vivo* by regulating the Akt/GSK-3β/cyclin D1 (protein kinase B/glycogen synthase kinase 3/cyclin D1) signaling pathway (Xiaoming *et al.* 2013).

The presence and relevance of βKL in normal and cancerous thyroids has not yet been investigated. In our previous study we demonstrated for the first time that a disrupted FGF19/FGFR4/βKL signaling pathway may play a role in the development of DTC and anaplastic thyroid cancers (Motylewska *et al.* 2018). This paper reports for the first time that serum concentration of βKL is decreased also in the neuroendocrine MTC. The mechanism of this effect needs further investigation. It is well known that cell-associated βKL acts as a coreceptor for FGFR4, which is required for high affinity binding and activity of FGF19. However, it is also assumed that βKL may be able to function in a similar manner to αKL, not only as cell-associated protein, but also as secreted extracellular portion of KL (Lin & Desnoyers 2012). Thus, βKL could potentially induced anticancer effects by modulating other signaling pathways.

The FGF-FGFR signaling network is involved in cell differentiation, migration and proliferation, as well as in morphogenesis and angiogenesis (Brooks *et al.* 2012; Turner & Grose 2010). Therefore, dysregulation in this signaling pathway seems to be important for oncogenesis. FGF19 is of particular interest in this issue, as it has been shown to play a role in metabolism under physiological conditions, but also in cancer development and progression. FGF19 signaling has been reported to be important in promoting hepatocellular (Desnoyers *et al.* 2008; Miura *et al.* 2012; Nicholes *et al.* 2002), colon (Desnoyers *et al.* 2008; Pai *et al.* 2008), gastric (Wang *et al.* 2016), prostate (Feng *et al.* 2013; Nagamatsu *et al.* 2015) and differentiated thyroid cancers (Zhang *et al.* 2016). Amplification of the *FGF19* gene was also reported by some authors in MTC (Heilmann *et al.* 2016). Surprisingly, FGF19 levels in patients diagnosed with MTC in our study did not differ significantly from those of healthy controls. However, we found FGFR4 serum concentration to be elevated in the MTC group in comparison to the control. This could be a consequence of increased production and expression of this growth factor in MTC tissues. In fact, as noted in another study, FGFR4 was the only FGF receptor type expressed in MTC TT cells and its pharmacological inhibition by PD173074 caused arrest of cell proliferation and tumor growth *in vivo* (Ezzat *et al.* 2005). FGFR4 also seems to play an important role in other thyroid cancers. FGFR4 was expressed predominantly in aggressive tumor types including 25% of follicular thyroid cancers (FTC), 23% of papillary thyroid cancers and 100% of poorly differentiated and anaplastic thyroid carcinomas. Also, in in vitro studies FGFR4 expression promoted DTC growth. Pharmacological FGFR4 tyrosine kinase inhibition resulted in the arrest of proliferation in an aggressive cell line endogenously expressing the receptor and induced FTC-derived cell growth inhibition in xenografted severe combined immunodeficient mice (St Bernard *et al.* 2005). Moreover, FGFR4 overexpression and polymorphisms (i.e., Gly388Arg) have been associated with a number of other cancers, including colorectal cancer, prostate carcinoma, lung cancer, squamous cell carcinoma, melanoma and soft tissue sarcoma (Lin & Desnoyers 2012). According to some authors, the activation of ERK phosphorylation could be implicated in these FGFR4-related carcinogenesis (Roidl *et al.* 2009).

In summary, our results reveal elevated IGF1R and FGFR4 signaling and loss of function by βKL in patients with MTC. This knowledge about C cell oncogenic transformation and MTC biology could be useful in developing effective targeted therapy in tis tumor.

CONCLUSION

Our findings indicate that IGF1R, FGFR4 and βKL signaling pathway dysfunction may play a role in medullary thyroid cancer development. However, further studies are required to confirm these findings and to use this knowledge in clinical practice.

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DECLARATION OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this paper.

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