Fibrotic response in fibroblasts from congenital disorders of glycosylation

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Abstract

Congenital disorders of glycosylation (CDG) are characterized by a generalized underglycosylation of proteins. CDG is associated with multiple symptoms such as psychomotor retardation, hypotonia, hormonal disturbances, liver fibrosis and coagulopathies. The molecular basis of these symptoms is poorly understood considering the large extent of affected glycoproteins. To better understand the cellular responses to protein underglycosylation in CDG, we have investigated the differences in gene expression between healthy control and CDG fibroblasts by transcriptome comparison. This analysis revealed a strong induction of several genes encoding components of the extracellular matrix, such as collagens, COMP, IGFBP5 and biglycan. The extent of this response was confirmed at the protein level by showing increased production of collagen type-I for example. This fibrotic response of CDG fibroblasts was not paralleled by a differentiation to myofibroblasts and by increased TGF-β signalling. We could show that the addition of recombinant IGFBP5, one of the induced proteins in CDG, to healthy control fibroblasts increased the production of collagen type-I to levels similar to those found in CDG fibroblasts. The fibrotic response identified in CDG fibroblasts may account for the elevated tissue fibrosis, which is often encountered in CDG patients.

Keywords: CDG • IGFBP5 • collagen • fibroblast • transcriptome

Introduction

N-linked glycosylation is a widespread and essential modification of proteins in eukaryotes. Oligosaccharides are first assembled on the lipid-carrier dolichol and then transferred co-translationally to asparagine residues of nascent glycoproteins [1, 2]. Loss or gain of individual N-glycosylation sites can alter the stability and function of the corresponding glycoprotein. Along this line, some forms of metachromatic leukodystrophy [3], thrombophilia [4, 5] and α1-antitrypsin deficiency [6] are caused by mutations leading to the loss of N-glycosylation sites. Alternatively, the gain of a N-glycosylation site in the IFNγ R2 protein has been shown to abolish IFNγ response in a form of immunodeficiency [7]. By contrast, defects of lipid-linked oligosaccharide (LLO) biosynthesis cause generalized decreased N-glycosylation sites occupancy due to limited availability of complete LLO in the endoplasmic reticulum (ER) leading to pleiotropic changes. In addition, the structure of N-glycans on glycoproteins can be defective when glycan processing and elongation steps are altered in the secretory pathway. Both forms of N-glycosylation defects belong to the family of congenital disorders of glycosylation (CDG) [8–10]. Considering the broad impact of glycosylation on protein functions, CDG are associated with developmental delay, malformations and multiple organ dysfunctions [11]. Although symptoms such as coagulopathies, gastrointestinal bleeding and protein-losing enteropathy are seen in specific types of CDG, hepatic and tissue fibrosis is a common feature observed in several forms of CDG. In some CDG cases, skin abnormalities such as cutis laxa [12] and ichthyosis [13] have also been described. However, the molecular mechanisms underlying most CDG symptoms are largely unclear [14].

Alterations of N-glycosylation affect multiple signalling pathways by altering the stability of membrane proteins or the signalling ability of membrane receptors [15]. For example, the loss of specific N-glycan branches increases the endocytosis of the glucose transporter GLUT2 [16] and of several cytokine receptors [17]. Similarly, changes of N-glycan core fucosylation inhibits...
Transforming growth factor-β (TGF-β) signalling, which leads to decreased production of extracellular matrix (ECM) proteins and to lung emphysema in p1–6 fucosyltransferase (FUT8) deficient mice [18]. Increased core fucosylation of N-glycans has also been observed in CDG cases with defects in the LLO synthesis pathway [19, 20], whereas the cytokine TGF-β observed in CDG cases with defects in the LLO synthesis pathway [21], additional proteins, such as factors of the CCN family [22] and the matricellular protein SPARC [23], have been reported to also mediate a profibrotic effect.

To gain insight into the pathogenesis of CDG manifestations, we have investigated the gene expression signature of CDG fibroblasts from patients with defects in the LLO synthesis pathway namely in DPM1 dolichylphosphate mannosynanse (DPM1-CDG, CDG-Ie) [24, 25], ALG12 mannosyltransferase (ALG12-CDG, CDG-Ig) [26–28] and ALG6 glucosyltransferase (ALG6-CDG, CDG-Ic) [29]. These three gene defects lead to the specific accumulation of the distinct LLO structures dolichyl-pyrophosphate-GlcNAc2Man8, dolichyl-pyrophosphate-GlcNAc2Man7 and dolichyl-pyrophosphate-GlcNAc2Man6g, respectively. Our study demonstrated an increased expression of several ECM proteins in CDG and pointed to the involvement of insulin-like growth factor-binding protein (IGFBP) 5 in the mediation of this fibrotic response.

Materials and methods

Cell culture

Primary fibroblasts from three healthy control subjects and from nine CDG patients were isolated from a skin biopsy and were grown in DMEM (Invitrogen, Basel, Switzerland) with 4.5 g/l glucose and 10% foetal calf serum. The CDG cells used have mutations in the dolichylphosphate mannosynanse (R92G/del628c; S257P/S257P; R92G/13-bp del), ALG12 mannosyltransferase (A81T/A81T; T67M/R146Q; G101R/G101R) and ALG6 glucosyltransferase (A333V/A333V; S478P/S478P; A333/IV33+5G>A) genes.

Collagen determination

Collagen was precipitated by the Sircol dye reagent and collected by centrifugation whereas the unbound dye was removed by adding 0.5 M NaOH. The Collagen concentration was determined performed with the Sircol assay reagent (Biocolor, Carrickfergus, UK) according to the manufacturer instructions. Fibroblasts (2 × 10^6) were digested overnight in 100 μl of pepsin (0.1 mg/ml; Sigma-Aldrich, Buchs, Switzerland) in Hanks Balanced Salt Solution, pH 7.0. Collagen was precipitated by the Sircol dye reagent and collected by centrifugation whereas the unbound dye was removed by adding 0.5 M NaOH. The absorption at 540 nm was measured by spectrophotometry.

Oligonucleotide arrays

Total RNA was isolated from fibroblast cultures and reverse transcribed as described previously [30]. cDNA samples were purified by phenol–chloroform extraction and 5 μl were in vitro transcribed in presence of biotin labelled nucleotides performed with the High Yield Transcription kit (Enzo Lifesciences, Lausen, Switzerland). Biotin-labelled cRNA samples (15 μg) were fragmented at 95 °C in 40 mM Tris-acetate, 100 mM K-acetate, 30 mM Mg-acetate and mixed in 300 μl of hybridization buffer containing a hybridization control mix, including reference genes (GAPDH, ACOT7), spike controls (BIOB, BIOC, CREX, BIODN) and proprietary Affymetrix B2 oligonucleotides, 0.1 mg/ml herring sperm DNA and 0.5 mg/ml acetylated bovine serum albumin in 2-(4-morpholino)-ethane sulfonic acid (MES) buffer, pH 6.7. HG-U133A gene chips (Affymetrix, High Wycombe, UK) were incubated with the cRNA samples for 16 hrs at 45 °C, and washed performed with an Affymetrix Fluidics Station 400. cRNA bound to the oligonucleotide arrays was stained with phycocerythrin-streptavidin and with biotin-labelled anti-streptavidin antibody. The fluorescent signal emitted by the labelled targets was measured performed with a Gene-Array scanner G2500 (Agilent, Basel, Switzerland). The array data is available at the GEO repository under accession number GSE8440.

Statistical analysis

Raw data processing was performed using the Affymetrix Microarray Suite Ver. 5.0 (MASS) software. After hybridization and scanning, probe cell intensities were calculated and summarized for the respective probe sets performed with MASS software [31]. The trimmed mean of each chip was normalized to a target intensity of 500 as recommended by Affymetrix. Scaling factors and appropriate numbers of present calls (≥50%) were calculated by application of a signed-rank call algorithm [32]. Statistical parametric methods based on the comparisons between the triplicates of each condition were applied. The Cross-Gene Error Model from the Gene Spring software 5.1 (Agilent) was applied to filter unreliable genes and unequal variance t-test for two, or ANOVA for more than two groups, were applied to detect differentially expressed genes. The False Discovery Rate was applied [33] to reduce the number of false-positive genes.

Immunofluorescence microscopy

Cells were fixed in 3.5% paraformaldehyde for 10 min. and permeabilized with 0.1% saponin in phosphate buffered saline (PBS). Cells were incubated with primary antibodies for 1 hr in 0.1% saponin in PBS. After washing twice with PBS, the cells were incubated with Alexa488-conjugated secondary antibody in 0.1% saponin in PBS for 30 min. Immunofluorescence images were taken by an Axiovert 200M microscope (Zeiss, Feldbach, Switzerland) performed with the Axiovision 3.0 software (Zeiss). The antibodies used were specific to collagen type-I (Sigma, clone COL-1), α-smooth muscle actin (Sigma, clone 1A4) and Smad4 (Santa Cruz Biotechnology, Heidelberg, Germany).

Western blot analysis

Proteins from the post-nuclear supernatants of fibroblast cells were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes according to Towbin et al. [34]. Membranes were incubated over night with a horseradish peroxidase conjugated antisem (Biocolor, Carrickfergus, UK) according to the manufacturer instructions. Fibroblasts (2 × 10^6) were digested overnight in 100 μl of pepsin (0.1 mg/m; Sigma-Aldrich, Buchs, Switzerland) in Hanks Balanced Salt Solution, pH 7.0. Collagen was precipitated by the Sircol dye reagent and collected by centrifugation whereas the unbound dye was removed by adding 0.5 M NaOH. The absorption at 540 nm was measured by spectrophotometry.

Western blot analysis

Proteins from the post-nuclear supernatants of fibroblast cells were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes according to Towbin et al. [34]. Membranes were incubated overnight with primary antibodies against IGFBP5 (Cell Signalling Technology, Alleschwil, Switzerland), COMP (Kamya Biomedical Company, Seattle, WA, USA), PSG1 (clone I110; BD Biosciences, Basel, Switzerland), endoglin (BD Biosciences, clone 35), β-tubulin (Sigma, clone SAP-465) and pSMAD2 (Cell signalling). After washing in Tris-buffered saline with 0.1% Tween-20, membranes were incubated with a horseradish peroxidase conjugated antisem.
secondary antibody (Sigma) for 1.5 hrs and signals were detected by chemiluminescence (Pierce, Lausanne, Switzerland).

**IGFBP5 and TGF-β treatment**

Fibroblasts from CDG patients and healthy controls were seeded at a density of 10^6 cells in 10 cm plates 24 hrs prior treatment with 10 ng/ml TGF-β (Sigma). Treatment with TGF-β was carried out for 36 hrs prior harvesting for Sircol assay. For the TGF-β sensitivity experiment, 0.2 × 10^6 fibroblasts were seeded in six-well plates overnight. Medium was exchanged with serum free medium for 10 min. before adding TGF-β in different concentrations for 30 min. to the cells. The reaction was stopped by washing the cells with ice cold PBS and cells were harvested and lysed for Western blot analysis. For IGFBP5 treatment, 0.2 × 10^6 healthy control fibroblasts were plated in six-wells on cover slips. Recombinant human IGFBP5 was given to the medium in the concentrations 0, 0.25, 0.75 and 1.5 μg/ml for 60 hrs prior to immunofluorescence.

**Results**

To appreciate the range of cellular responses linked to glycosylation disorders, we have analysed the global gene transcription profile of ALG6-CDG, DPM1-CDG and ALG12-CDG fibroblasts by oligonucleotide array hybridization [30]. Genes with similar expression profiles in the different CDG were resolved by hierarchical clustering. This analysis showed that multiple genes encoding components of the extracellular matrix were highly induced in the three forms of CDG (Fig. 1). The induced genes comprised collagen genes such as COL1A1, COL4A1, COL4A2, COL5A1, COL11A1 and COL1A1, cartilage oligomeric protein (COMP), biglycan (BGN), hyaluronan and proteoglycan link protein 1 (HAPLN1), fibronectin (FN), tenascin-C (TNC) and nidogen-1 (NID1) in particular (Table 1). Matrix metalloproteinases, such as the BMP1 collagenase and the MMP3 proteoglycanase were also among the genes significantly overexpressed in CDG fibroblasts. Another group of ECM genes induced in CDG fibroblasts were four members of the pregnancy-specific glycoproteins (PSG). PSG are closely related to the carcinoembryonic antigen family and are mainly transcribed in the placenta [35], although they are also expressed in other tissues according to the UniGene’s EST Profile Viewer [36]. Components of the TGF-β pathway were also found among the list of genes induced in CDG. The TGF-β and TGF-β receptor-I mRNAs were slightly elevated in CDG fibroblasts whereas endoglin, a co-receptor for TGF-β [37], was induced between 1.9- and 3.6-fold in CDG fibroblasts (Table 1). Members of the Wnt/β-catenin signalling pathway such as the Wnt2

Fig. 1 Gene clustering analysis of ECM genes. The colours indicate the expression levels relative to the median of all measurements for the gene throughout all the samples analysed. Red means up-regulated and green means down-regulated in comparison to the median. The blue arrows mark collagen genes and the red arrows other ECM genes.
Table 1 mRNA levels of ECM genes in CDG fibroblasts

| Gene                                   | Symbol | Accession no. | ALG6-CDG | DPM1-CDG | ALG12-CDG |
|----------------------------------------|--------|---------------|----------|----------|-----------|
| Collagen type 1, α1                    | COL1A1 | BC036531      | 3.6      | 7.2      | 5.5       |
| Collagen type 4, α1                    | COL4A1 | NM_001845     | 1.3      | 2.6      | 2.3       |
| Collagen type 4, α2                    | COL4A2 | NM_001846     | 1.3      | 2.0      | 2.9       |
| Collagen type 5, α1                    | COL5A1 | NM_000093     | 1.8      | 2.1      | 1.5       |
| Collagen type 11, α1                   | COL11A1| NM_001854     | 3.3      | 10.3     | 11.9      |
| Collagen type 18, α1                   | COL18A1| NM_0030582    | 1.5      | 2.1      | 2.0       |
| Cartilage oligomeric matrix protein    | COMP   | NM_000095     | 18.4     | 21.1     | 8.0       |
| Hyaluronan and proteoglycan link protein-1| HAPLN1 | NM_001884     | 5.7      | 2.1      | 17.2      |
| Biglycan                               | BGN    | NM_001711     | 4.4      | 5.1      | 2.8       |
| Fibronectin                            | FN1    | NM_212482     | 1.9      | 3.8      | 2.8       |
| Thrombospondin-1                       | THBS1  | AI812030      | 1.4      | 4.4      | 1.8       |
| Tenasin-C                              | TNC    | NM_002160     | 2.4      | 1.6      | 1.0       |
| Nidogen-1                              | NID1   | NM_002508     | 1.7      | 2.2      | 1.0       |
| Tissue factor pathway inhibitor-2      | TFF12  | NM_006528     | 3.2      | 2.2      | 0.3       |
| EGF-like repeats and discoidin I-like domains-3| EDIL3 | NM_005711     | 4.4      | 6.1      | 7.7       |
| Bone morphogenic protein-1             | BMP1   | NM_001199     | 2.1      | 2.0      | 2.1       |
| Matrix metalloproteinase-3             | MMP3   | NM_002422     | 1.3      | 3.6      | 1.7       |
| Pregnancy-specific glycoprotein-1      | PSG1   | NM_006905     | 6.2      | 5.0      | 5.7       |
| Pregnancy-specific glycoprotein-4      | PSG4   | NM_002780     | 5.3      | 4.0      | 5.4       |
| Pregnancy-specific glycoprotein-6      | PSG6   | NM_002782     | 1.4      | 1.2      | 1.4       |
| Pregnancy-specific glycoprotein-7      | PSG7   | NM_002783     | 6.6      | 7.3      | 7.0       |
| Insulin-like growth factor binding protein-5 | IGFBP5 | R73554     | 8.1      | 3.0      | 1.5       |
| Neuroplin-2                            | NR2P   | NM_201266     | 1.7      | 3.0      | 2.2       |
| Interleukin-8                          | IL8    | NM_000584     | 2.4      | 5.5      | 1.4       |
| Transforming growth factor-β           | TGFβ1  | NM_00316       | 1.2      | 1.9      | 2.0       |
| TGF-β receptor-1                       | TGFBR1 | NM_004612     | 1.6      | 1.9      | 1.3       |
| Endoglin                               | ENG    | NM_000118     | 1.9      | 3.6      | 2.7       |
| Homolog of mothers against decapentaplegic, 7 | SMAD7 | NM_005904     | 1.4      | 1.9      | 0.8       |
| Wingless-type MMTV integration site family, member 2 | WNT2 | NM_003391     | 6.1      | 2.3      | 2.5       |
| WNT1-inducible signalling pathway protein 1 | WISP1 | AB034725     | 5.5      | 7.0      | 5.0       |
| SRY-box 11                             | SOX11  | AB028641      | 6.3      | 5.6      | 5.4       |

The values represent averages of independent measures and indicate the fold-induction compared to the mRNA levels measured in control cells. Fibroblasts from four ALG6-CDG, three DPM1-CDG and two ALG12-CDG cases were investigated. The expression values obtained in the CDG fibroblasts were significantly different from the control values (one-way ANOVA-test, P < 0.05).

signalling protein (WNT2), the Wnt-1 inducible signalling pathway protein (WISP-1) as well as the Sox11 transcription factor were overexpressed above twofold in CDG fibroblasts.

To validate the up-regulation of ECM components at the protein level, we first measured collagen production in fibroblasts. Using the Sircol dye assay, we detected elevated collagen concentrations in all CDG fibroblasts tested (Fig. 2A). The increased collagen production was confirmed by immunofluorescence analysis of fibroblasts performed with an antibody to collagen type-I (Fig. 2B). The induction of ECM components in CDG was also confirmed by
Western blot analysis of the proteins IGFBP5, COMP, endoglin and PSG1 (Fig. 3), whereas the production of ECM proteins by fibroblasts is often associated with myofibroblastic differentiation, such a phenotype was not observed in CDG fibroblasts, as assessed by the low level of the myofibroblast marker protein α-smooth muscle actin [38] (data not shown).

The cytokine TGF-β is a prominent stimulus of ECM expression [21]. To examine whether CDG fibroblasts are more sensitive to TGF-β than control fibroblasts, we treated CDG and control fibroblasts with the profibrotic cytokine TGF-β. The levels of collagen production were strongly increased in all treated cells with the exception of DPM1-CDG fibroblasts (Fig. 4). We also investigated whether CDG fibroblasts are more sensitive to TGF-β, considering the increased expression of endoglin in those cells. The levels of SMAD2 phosphorylation were measured in control and CDG fibroblasts stimulated with increasing amounts of TGF-β.

This titration showed no clear difference of SMAD2 phosphorylation between control and CDG fibroblasts (Fig. 5). Finally, we investigated the activation of the TGF-β pathway by examining the level of SMAD4 nuclear localization in CDG and control fibroblasts. Also this parameter did not differ significantly between the cells examined (data not shown), indicating that the TGF-β pathway did not account for the increased ECM protein expression in CDG fibroblasts.

The observed increased expression of IGFBP5 in CDG was unexpected. The IGFBP5 protein has been associated with pulmonary fibrosis, where IGFBP5 has been shown to stimulate the production of ECM proteins by activating fibroblasts [39]. Therefore, we did test whether the increased IGFBP5 expression in CDG fibroblasts could be related to the increased production of ECM components in these cells. We incubated control fibroblasts with recombinant IGFBP5 at increasing concentrations. The addition of IGFBP5 at 0.25 μg/ml was sufficient to increase the level of collagen production after 2 days, whereas this effect was highest at an IGFBP5 concentration of 1.5 μg/ml (Fig. 6). This effect was similar to that obtained when stimulating fibroblasts with 10 ng/ml of TGF-β.

Overall we showed that decreased N-glycosylation site occupancy parallels the expression of ECM genes. The unchanged TGF-β signalling pathway in control and patient cells pointed to a different molecular mechanism of ECM induction. The fact that it was possible to increase the collagen expression by recombinant IGFBP5 in controls showed that this protein is probably involved in the induction of ECM gene expression in CDG.

Discussion

N-linked glycosylation is required for the proper function of many glycoproteins. The stability of membrane proteins is depending on the branching of N-glycans and the degree of N-glycosylation site occupancy [15]. CDG with defects of LLO synthesis pathway leads to unoccupied N-glycosylation sites. To address the physiological processes and molecular pathways altered by deficient N-glycosylation site occupancy, a global gene transcription array study was performed in different CDG subtypes. In an earlier study, we could show that the unfolded protein response and amino acid metabolism were induced in CDG fibroblasts [30]. In this work, we have shown that pro-fibrotic genes were also induced in CDG fibroblasts. This observation fits with the observation of tissue fibrosis in some types of CDG [40–43].

The overexpression of COMP in CDG fibroblasts may be related to the interaction of this protein with some types of collagen, such as collagen type-I, which was also overexpressed in CDG fibroblasts. COMP expression has been shown to be TGF-β dependent in systemic sclerosis [44], atherosclerotic lesions [45] and chronic pancreatitis [46]. Altered folding and secretion of COMP causes pseudoachondroplasia [47], but changes of COMP expression have not been associated with pathogenic phenotypes yet.
Identifying the underlying molecular reasons for the up-regulation of ECM genes can provide clues for potential therapeutic targets. We first tested the TGF-β signalling pathway as the possible actor. Although some members of the TGF-β signalling pathway were among the induced genes, the whole pathway was not induced in CDG and also the sensitivity of the pathway was not altered. Therefore, a second agent, IGFBP5 that is less well characterized than TGF-β was investigated as a booster of ECM gene expression (Fig. 5). Stimulation of control fibroblasts with IGFBP5 indeed showed a similar effect of collagen induction as the stimulation with TGF-β. This observation could be extended to CDG fibroblasts (Fig. 6). The IGFBP5 protein has previously been associated to fibrosis. IGFBP5 is over-expressed in systemic sclerosis fibroblasts and in idiopathic pulmonary fibrosis [39, 48]. Adenoviral expression of IGFBP5 in skin of mice induced fibrosis with increased dermal thickness and increased deposition of collagen and fibronectin [49]. IGFBP5 is proposed to be a central player in the initiation of fibrosis and in the response to injury [49, 50]. However, the molecular basis of
IGFBP5-mediated fibrosis is not yet completely understood. Members of the family of the IGFBPs bind with high affinity to the insulin-like growth factors (IGF)-I and -II and therefore regulate the activity of the IGF-axis either positively or negatively [51, 52]. The IGF axis is a key regulator of cellular growth, differentiation and apoptosis processes [53]. Probably the more relevant mechanism for the ECM induction by IGFBP5 is the ability of IGFBP5 to bind to ECM components such as collagens, fibronectin and heparan sulphates [54, 55]. This binding may protect the ECM proteins from degradation and overexpression of IGFBP5 causes the deposit of big amounts of ECM proteins. The binding of IGFBP5 to ECM proteins also deprives the pool of free IGFBP5 that can bind IGFs and therefore has an influence on the activity of the IGF pathway [49, 56].

The reasons why protein underglycosylation goes along with the up-regulation of IGFBP5 expression are unknown and we can only speculate. Receptor activities are influenced by altered glycosylation. Besides the TGF-β signalling pathway also cytokine receptors are influenced by glycosylation. The overexpression of β1,6-N-acetylglucosaminyltransferase-V (MGAT5), which initiates the fourth branch of N-glycans, is leading to a sensitization of the cells to cytokines due to retarded endocytosis of the receptors [17]. This sensitization was depending on the branching of the N-glycan, hence the MGAT5 expression level, but also on the number of total N-glycans. Therefore, underglycosylation due to a deficient LLO synthesis pathway may alter cytokine response and thus the downstream targets as, for example IGFBP5.

The correlation between the underglycosylation of proteins in CDG patients and the observed symptoms are poorly understood. Appreciating and understanding the mechanisms underlying the symptoms represent a first step towards the identification of potential targets for therapeutic approaches. Hepatic fibrosis creates a long-term risk for CDG patients and represents a negative prognostic factor for the application of mannose therapy in CDG-Ib [42]. By comparison, fibrotic changes in systemic sclerosis fibroblasts reflect a generalized deposition of ECM proteins also in internal organs [57]. Although TGF-β, integrin/ILK and Wnt/β-catenin pathways represent the three major signalling pathways driving tissue fibrosis, ECM deposition can be induced by different stimuli [58]. We have identified IGFBP5 as a potential inducer of collagen type-I in CDG-fibroblasts. Future efforts in characterizing the IGFBP5-dependent fibrotic phenotype will help to identify the fine tuning of molecules driving liver fibrosis in CDG and to develop disease-specific therapeutic targets.

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Conflict of interest

The authors declare no conflict of interest.
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