Cyclin D1 Overexpression versus Response to Induction Chemotherapy in Squamous Cell Carcinoma of the Head and Neck

Preliminary Report

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Acta Oncologica Vol. 40, No. 4, pp. 505–511, 2001

The aim of this study was to investigate whether there is an association between overexpression of cyclin D1 and response to therapy. Immunohistochemical overexpression of cyclin D1 was determined in paraffin-embedded specimens from diagnostic biopsies of 89 primary cases of squamous cell carcinoma of the head and neck (SCCHN), using a polyclonal antiserum. The tumor response rates were estimated after curative treatment (i.e. surgery and/or radiotherapy and/or chemotherapy). Patients whose tumors were overexpressing cyclin D1 showed complete or partial response to neoadjuvant chemotherapy with cisplatin/5-FU. In addition, a majority of cyclin D1 negative tumors did not respond at all to this treatment ($p = 0.02$, Fisher's exact test). This study indicates that immunohistochemical assessment of cyclin D1 expression in SCCHN could be a new predictive marker to select a subgroup of patients that will benefit from neoadjuvant chemotherapy.

Received 4 January 2000
Accepted 15 November 2000

Neoadjuvant chemotherapy with cisplatin and 5-fluorouracil (5-FU) was introduced for treatment of squamous cell carcinoma of the head and neck (SCCHN) in the early 1980s. Complete response rates up to 30% were initially reported (1, 2), as well as a decreased frequency of distant metastases (3–5). However, in a recently performed meta-analysis by Pignon et al. (MACH-NC Collaborative group) of 63 trials, including 10 717 patients, very little benefit in survival data was reported for any chemotherapy treatment and probably none at all for neoadjuvant chemotherapy (pers. comm.). Other series show even poorer outcomes for treated patients (6). A possible reason for these latter findings might be that non-responders have to wait up to 7 weeks until effective treatment (i.e. radiotherapy or surgery) is initiated (6, 7). In addition, physicians have to consider the risks of side effects, such as nephro-, neuro-, and myelotoxicity (cisplatin) (8), and myocardial toxicity (5-FU) (9). This problem is especially serious for non-responders, whereas for responders the risks could be worth the benefit derived from therapy.

Several different markers to predict response to neoadjuvant therapy with cisplatin/5-FU have been suggested over the years, e.g. low levels of C1q binding molecules (10), as a sign of low humoral immunity, aneuploidy by flow cytometry (11, 12), and p53 overexpression (13), with all three parameters correlating with a good response in these studies. However, to our knowledge none of them have been tested in clinical, prospective trials.

Cisplatin is an effective anticancer drug with alkylating properties (8). The antitumor activity is stated to be achieved by binding of the drug to DNA, and the formation of adducts to DNA resulting in crosslinks which inhibit DNA replication and the transcription process, which causes a block in G2 phase or in the S to G2 transition. Cisplatin affects solely DNA synthesis (Fig. 1), while RNA and protein synthesis are left unaffected (8). 5-FU is also thought to act mainly in the S-phase of the cell cycle (Fig. 1), as the drug is incorporated into DNA during DNA synthesis, acting as an antimetabolite, and thus disturbing normal cell proliferation (9). Combined treatment with cisplatin and 5-FU has a supra-additive antitumor effect on DNA compared to treatment with single drugs (14).

Cyclin D1 is a cell cycle regulating protein, involved in the G1 to S transition (Fig. 1). The gene, *CCND1*, is located at chromosome 11, band q13. It is a potential

 ISSN 0284-186X

proto-oncogene as it is amplified as well as overexpressed in a number of human cancer types, e.g. breast (15), esophageal cancer (16, 17), and SCCHN (18, 19). In SCCHN, about 50–60% of the tumors overexpress cyclin D1 (19–21), and overexpression of the protein correlates with tumor progression (16) and poor prognosis (19, 20).

The aim of our study was to investigate whether cyclin D1 overexpression is associated with response to induction chemotherapy and/or primary radiotherapy.

MATERIAL AND METHODS

Patients and tumors

The series comprised 89 patients with single primary SCCHN who had been the subjects for an earlier report (19). Tumor samples were obtained by diagnostic biopsy or during primary surgery in the period 1987 through 1991. All tumors were classified according to the International Union Against Cancer criteria (22). None of the patients had received treatment prior to biopsy, and all received treatment with curative intent after diagnosis.

The following sites were represented: oral cavity ($n = 36$), oropharynx ($n = 22$), larynx ($n = 19$), hypopharynx ($n = 9$), maxillar sinus ($n = 2$) and epipharynx ($n = 1$). Forty-four (49%) of 89 tumors were T3-4 and 35/89 (39%) had lymph node metastases at the time of diagnosis.

Neoadjuvant chemotherapy

The inclusion criteria were the presence of unresectable locoregional SCCHN, stages II to IV (22), and the World Health Organization performance status 2 or less. Patients with impaired hearing or renal function (serum creatinine level, > 130 μmol/L [> 147 mg/dL]) or with cardiac disease that did not allow hydration during the chemotherapy were excluded.

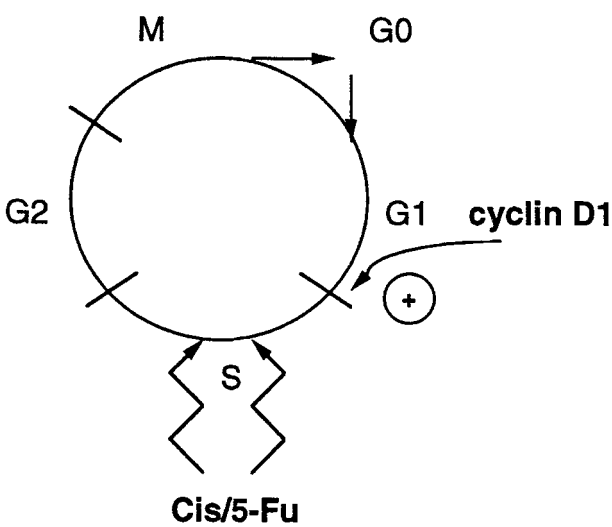

Fig. 1. The cell cycle regulating protein cylin D1 is involved in the G1 to S-phase transition. Tumors overexpressing the protein have a higher proportion of the cells in S-phase, where they are sensitive to treatment with cisplatin/5FU.

Twenty-eight of 89 (31%) patients were given cisplatin/5-FU as induction chemotherapy. The treatment was administered as follows: cisplatin 100 mg/m^2 on day 1 and subsequent infusions of 5-FU 1000 mg/m^2 on days 1–5, repeated every 3 weeks, for a total of 3 cycles. Heart, kidney and hearing functions, as well as blood count, were routinely controlled prior to chemotherapy. Twelve of the 28 patients did not complete all three cycles, owing to adverse effects and intercurrent diseases (e.g. myocardial infarction, deteriorating clinical performance, deranged electrolytes, hearing loss) and 2 patients were never evaluated for tumor response.

Thus, response to induction chemotherapy could be assessed in a total of 14 patients (Table 1). The following sites were represented: oral cavity ($n = 6$), oropharynx ($n = 5$), larynx ($n = 2$) and hypopharynx ($n = 1$). Twelve of 14 (86%) patients had T3 or T4 tumors, and lymph node metastases were seen in 8/14 (57%) patients. Mean age at diagnosis was 67.5 years (range 59.0–78.1). Treatment of one of the patients was switched from cisplatin/5-FU to carboplatin/5-FU after the first cycle because of impaired renal function (case no. 3).

The 14 patients who did not complete all three cycles or were not evaluated had tumors that originated in the following sites: hypopharynx ($n = 5$), oral cavity ($n = 4$), oropharynx ($n = 3$) and larynx ($n = 2$). Nine of these 14 patients (64%) had T3 or T4 tumors and 5/14 (36%) showed lymph node metastases at the time of diagnosis.

Radiotherapy (RT)

Forty of 89 (45%) patients received primary RT, which was given after 3D planning with a linear accelerator, 4–6 MV photons ($n = 25$), or with cobolt 60 ($n = 15$), at 2 Gy/fraction, 5 fractions/week, up to a total absorbed dose of 64–70 Gy to the primary tumors, and 50 Gy preoperatively to lymph node metastases. In some cases metastatic lymph nodes were included in the field receiving full-dose RT. Twenty of 40 (50%) patients had T3-4 tumors, and 14/40 (35%) showed lymph node metastases. The following sites were represented in this group: oral cavity ($n = 11$ cases), oropharynx ($n = 12$), epi- and hypopharynx (one each), and larynx ($n = 15$). One of the patients was not evaluated for response to RT.

Surgery

Twenty-one of 89 (24%) patients with T1 or resectable T2-4 tumors underwent primary surgery. Sixteen of these 21 patients received preoperative RT. Those with regional metastases at the time of diagnosis were treated with neck dissection, usually after preoperative RT.

Clinical evaluation

Response to therapy, assessed about 4 weeks after completed chemotherapy and 5–6 weeks after RT, was evaluated by clinical examination, palpation, or endoscopy

Table 1

Overexpression of cyclin D1 correlates with response to neoadjuvant chemotherapy with Cisplatin/5-FU in patients with SCCHN (n = 14)

No.	Site	T	N	M	Cyclin D1	Response		
						T	N	M
1.	Oropharynx	3	0	0	−/+	CR		
2.	Floor of mouth	3	3	0	−/+	CR	CR	
3.	Oropharynx	4	2	0	−/+	PR	PR	
4.	Floor of mouth	4	3	0	−/+	PR	CR	
5.	Oropharynx	2	0	0	+	PR		
6.	Oropharynx	2	0	0	+	PR		
7.	Hypopharynx	4	2	0	++	PR	NR	
8.	Floor of mouth	3	0	1	–	PR		NR
9.	Larynx	3	0	0	–	PR		
10.	Floor of mouth	3	2	0	–	NR	PR	
11.	Oropharynx	3	2	0	–	NR	CR	
12.	Floor of mouth	4	1	0	–	NR	PR	
13.	Trig. Retromolar	3	1	0	–	NR	NR	
14.	Larynx	3	0	0	–	NR		

N, No stained tumor cells, −/+, 0–5% of the tumor cells are positive; +, 5–50%; ++, >50%. Abbreviations: NR = No response; PR = Partial response; CR = Complete response; SCCHN = Squamous cell carcinoima of the head and neek.

under anesthesia. Complete response (CR) was defined as no macroscopic disease at clinical evaluation, partial response (PR) as > 50% macroscopic reduction of the tumor diameter, and no response (NR) as < 50% reduction, or < 25% increase, of the tumor diameter.

Immunohistochemistry (IHC)

The procedures for producing antibodies against cyclin D1, and for IHC staining have been described in detail earlier (20). Briefly, an antiserum against cyclin D1 was generated by injection of a β-galactosidase-cyclin-D1 fusion protein into rabbits. Antibodies directed against the cyclin D1 part of the fusion protein were affinity-purified. Antibodies reactive with β-galactosidase and bacterial (contamination) proteins were removed.

The affinity-purified polyclonal antibody was used for IHC, at a dilution of 1 : 80, using PBS/1% BSA. Tissue sections were incubated with the primary antibody for 16 h at 4°C and with the peroxidase-labeled conjugate for 30 min at room temperature. A two- stage streptavidin-biotin-peroxidase technique was used (Dako Duet kit; Dako, Glostrup, Denmark). Negative controls involved the omission of the antiserum from the primary incubation. A strongly cyclin D1 positive SCCHN specimen, kindly provided by Dr Francois Janot, Institute Gustave Roussy, Villejuif, France, served as positive control. In order to diminish the risk of false-negative findings owing to intratumor heterogeneity, serial sections were analyzed.

All IHC results were assessed by two independent observers (JÅ, MD), who were unaware of the clinical data.

IHC results were scored as follows: negative (−); 0–5% of the tumor cells positive (+/−); 5–50% positive (+); and > 50% positive (++), in accordance with earlier studies (19, 20).

Statistical analysis

Statistical analysis of the data was performed using True Epistat software (Epistat Services, Richardson, Tx, USA). Fisher's exact test was used to investigate differences in response to therapy between groups showing different IHC results. Survival curves were plotted by the Kaplan–Meier method, and differences were calculated with the log-rank test.

RESULTS

Tumor biopsies from 7 of 14 (50%) patients treated with neoadjuvant chemotherapy showed overexpression of cyclin D1 (Fig. 2). In general, the quality of the IHC staining procedure was good, only nuclei were stained and no cytoplasmic positivity was seen. A strongly positive specimen was used as a positive control. However, in the test series, all seven positive cases but one were rather weakly stained.

All 7 (100%) patients with cyclin D1 positivity at any level (+/−, + or ++) responded partially (PR) or completely (CR) to 3 cycles of cisplatin/5-FU (Table 1). Only 2 out of 7 (29%) patients with tumors that were immunohistochemically negative for cyclin D1 responded. The difference in response to therapy between cases overexpressing cyclin D1 at any level or not expressing the protein was significant ($p = 0.02$, Fisher's exact test) (Table

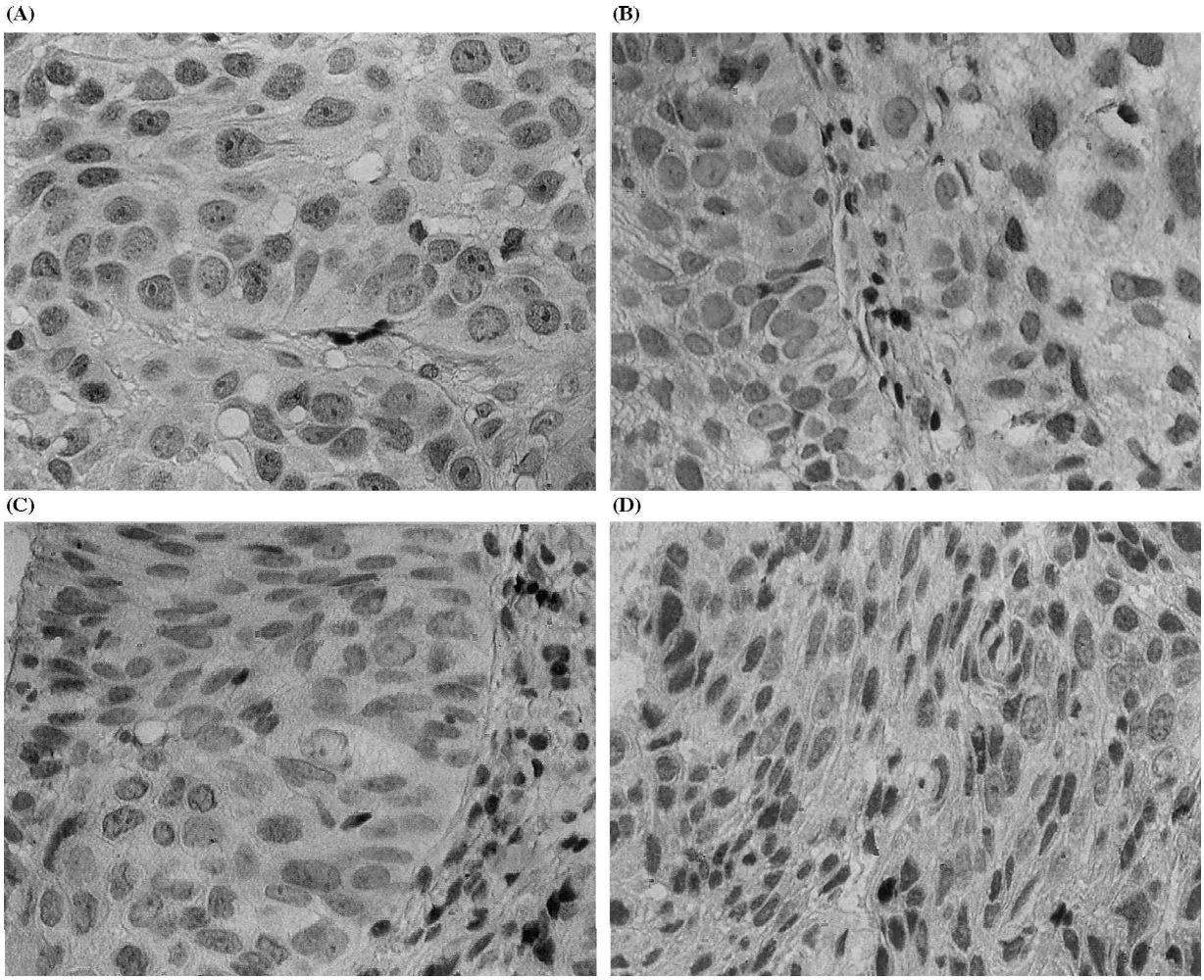

Fig. 2. Cyclin D1 overexpression in squamous cell carcinoma of the head and neck (SCCHN). A: ++ (>50% positive cells); B: + (5–50% positive cells); C: −/+ (0–5% positive cells); D: − (negative).

2). There were no differences in T status, metastatic spread or site between the two groups ($p = 0.56$, $p = 1.00$, $p = 0.10$, respectively [Fisher's exact test]), nor were there any differences in age between the groups ($p = 0.45$, Student's t-test).

There were no differences in T- or N status between the group of patients treated with 3 cycles of cisplatin/5-FU ($n = 14$) and the group of patients that did not complete all 3 cycles or were not evaluated after neoadjuvant chemotherapy ($n = 14$) ($p = 0.38$ and $p = 0.45$, respectively [Fisher's exact test]).

Forty of 89 (45%) patients received primary curative radiotherapy, one of whom had never been evaluated for response to therapy. No association was found between response to given radiotherapy and overexpression of cyclin D1 among the remaining 39 patients (Table 3).

Response to primary surgery vs. cyclin D1 overexpression could not be analyzed, as 16 of 21 (76%) patients in this group received preoperative RT up to 50 Gy.

Of the 5 patients who received only 2 cycles, 4 were evaluated regarding response to therapy, 2 of them showing CR. One of these patients was cyclin D1 positive (+) and the other negative. Inclusion of these 2 patients would not have influenced the statistical significance in the study group. The remaining 2 patients did not show any tumor response after the second cycle. Both of them were cyclin

Table 2

All of the 7 (100%) patients with cyclin D1 positivity at any level (−/+, + or ++) responded partially or completely to three given cycles of Cisplation/5-FU

Response to chemotherapy	Cyclin D1 expression	
	No	Yes
Complete/partial	2	7
No	5	0

$p = 0.02$, Fisher's exact test.

Table 3

No correlation was observed betweem cyclin D1 overexpression and response to primary radiotherapy in 39 SCCHN patients

Response to radlotherapy	Cyclin D1 expression	
	−	+
Complete	17	14
Partial	5	2
No	0	1

$p = 0.44$, Fisher's exact test.
Abbreviations: SCCHN = Squumons cell carinoma of the head and neek.

D1 positive (+ and + +, respectively). These two patients cannot be included, as they might have responded to a third cycle.

Among patients who received 3 cycles of induction chemotherapy ($n = 14$) there was a trend toward better survival ($p = 0.10$, log-rank test) for those who had cyclin D1-positive tumors compared with those with negative tumors (Fig. 3).

DISCUSSION

The results in the present study lead to the hypothesis that overexpression of cyclin D1, analyzed in diagnostic biopsies, may be a potential predictive marker for response to neoadjuvant chemotherapy with cisplatin/5-FU. However, as the number of patients in the study was limited, the study has to be defined as preliminary. A confirmatory study on a larger clinical material is presently being conducted, in order to verify the conclusions. Furthermore, based on this hypothesis we are presently testing a series of SCC cell lines for their chemosensitivity. Preliminary data from that series show a correlation between *CCND1* amplification, detected by real-time PCR, and sensitivity to cisplatin (data not shown).

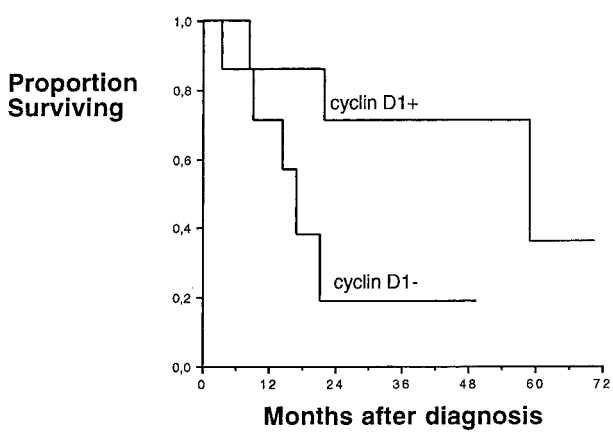

Fig. 3. Survival analyis of death from squamous cell carcinoma of the head and neck (SCCHN) among patients who received neoadjuvant chemotherapy ($n = 14$) shows a trend towards better survival in the subgroup of patients whose tumors were cyclin D1 positive compared with those whose tumors wer cyclin D1 negative ($p = 0.10$, log-rank test).

The biological basis for the correlation between cyclin D1 overexpression and response to induction chemotherapy with cisplatin/5-FU in the present study is reasonable. Cells overexpressing the protein expressed by the proto-oncogene will, in a higher proportion than those which do not overexpres it, continue their way through the cell cycle, pass the restriction point in G1 and go into S-phase (21) where they are vulnerable to treatment with cisplatin/5-FU, which interferes with DNA synthesis (23–27).

These preliminary results are in accordance with earlier findings showing better response to chemotherapy in flow cytometrically aneuploid cases compared with diploid cases (11, 12), as both markers are indicators of genetic instability. Cyclin D1 overexpression is a result of *CCND1* amplification and/or other genetic deregulation (19), e.g. translocations, and aneuploidy is the final result of abnormal cell-cycle regulation, especially in G2/M-phases, observed as hyper- or hypodiploid chromosome numbers.

Warenius et al. reported a correlation between high expression of cyclin D1 and cisplatin resistance in a series of human cell lines (28). However, it is difficult to compare these data with the findings in the present study as only three SCC/epidermoid cell lines were investigated, and the variability in cyclin D1 expression as well as in cisplatin sensitivity was low among these three cell lines.

In a previous study, based partly on the same patient material, we demonstrated a correlation between high levels of overexpression of cyclin D1 (+ +) and poor prognosis in SCCHN. These findings cannot be directly compared with the present data, as only one of the patients with cyclin D1 + + staining received induction chemotherapy. Logically, from a biological point of view, the strongly positive tumors ought to respond even better, a theory to be tested in further studies.

In the present study, 3 of 5 cyclin D1 negative cases responded partly or completely in regional or distant metastases (case Nos. 10–12, Table 1), a finding that could controverst our findings. However, we had no possibility to determine expression of cyclin D1 in the lymph nodes since, after neoadjuvant chemotherapy, these patients received preoperative radiotherapy prior to neck dissection.

In general, better specific prognostic markers are needed to identify patients at high risk for recurrence and shorter survival, regardless of the established parameters, e.g. the TNM classification system (29). However, contradictory results have often been reported from different investigators on such new markers. One reason for this low reproducibility is the great heterogeneity in most retrospective SCCHN materials, e.g. regarding tumor site and treatment (Table 4). An appropriate order for the investigations that will lead to the identification of a reliable prognostic marker might be, first, to show its ability to predict response to therapy and then test whether treatment strate-

Table 4

Argument for the use of markers that predict response to therapy rather than survival: A hypothetical example of two patients with identical survival rates, but totally different responses to radiotherapy

Patient	Treatment	Follow-up (24 months)
1. Tonsil, T2N0	RT: CR	NED
2. Tonsil, T2N0	RT: NR, salvage surgery; CR	NED

Abbreviations: RT = Radiotherapy; CR = Complete response; NR = No response; NED = No evidence of disease.

gies based on analysis of the marker really improve survival.

The hypothesis raised in the present study is that cyclin D1 overexpression, immunohistochemically detected in diagnostic biopsies or from operative specimens, might be a marker that can predict response to induction chemotherapy. The importance of such a predicitve marker is substantial when one considers that induction chemotherapy with cisplatin/5-FU is accompanied by a high frequency of toxicity. In order to prevent severe treatment-related morbidity in non-responders, it is important to select patients carefully.

In the present study there was a trend toward better survival for patients treated with neoadjuvant chemotherapy whose tumors were cyclin D1 positive compared with those with cyclin D1 negative tumors. Even taking into account that the number of patients in these groups was small, these findings indicate the potential of indivualized treatment on the basis of biological markers. However, whether the use of immunohistochemically detected cyclin D1 overexpression as a predicitive marker for induction chemotherapy in SCCHN will ultimately result in increased survival needs to be tested in a randomized prospective setting, in which identified cyclin D1 negative patients continue directly to other treatment modalities.

ACKNOWLEDGEMENTS

This study was sponsored by the Swedish Cancer Foundation, The King Gustaf V Jubilee Fund and Swedish Society for Medical Research.

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