

# Differences in food intake of tumour-bearing cachectic mice are associated with hypothalamic serotonin signalling

Jvalini T. Dwarkasing<sup>1</sup>, Mark V. Boekschoten<sup>2</sup>, Joseph M. Argilès<sup>3</sup>, Miriam van Dijk<sup>4</sup>, Silvia Busquets<sup>3</sup>, Fabio Penna<sup>3</sup>, Miriam Toledo<sup>3</sup>, Alessandro Laviano<sup>5</sup>, R. F. Witkamp<sup>1</sup>, & Klaske van Norren<sup>1,4\*</sup>

<sup>1</sup>Nutrition and Pharmacology Group, Division of Human Nutrition, Wageningen University, Wageningen, The Netherlands; <sup>2</sup>Nutrition, Metabolism and Genomics Group, Division of Human Nutrition, Wageningen University, Wageningen, The Netherlands; <sup>3</sup>Cancer Research Group, Departament de Bioquímica i Biologia Molecular, University of Barcelona, Barcelona, Spain; <sup>4</sup>Nutricia Research, Utrecht, The Netherlands; <sup>5</sup>Department of Clinical Medicine, Sapienza University, Rome, Italy

## Abstract

**Background** Anorexia is a common symptom among cancer patients and contributes to malnutrition and strongly impinges on quality of life. Cancer-induced anorexia is thought to be caused by an inability of food intake-regulating systems in the hypothalamus to respond adequately to negative energy balance during tumour growth. Here, we show that this impaired response of food-intake control is likely to be mediated by altered serotonin signalling and by failure in post-transcriptional neuropeptide Y (NPY) regulation.

**Methods** Two tumour cachectic mouse models with different food intake behaviours were used: a C26-colon adenocarcinoma model with increased food intake and a Lewis lung carcinoma model with decreased food intake. This contrast in food intake behaviour between tumour-bearing (TB) mice in response to growth of the two different tumours was used to distinguish between processes involved in cachexia and mechanisms that might be important in food intake regulation. The hypothalamus was used for transcriptomics (affymetrix chips).

**Results** In both models, hypothalamic expression of orexigenic NPY was significantly higher compared with controls, suggesting that this change does not directly reflect food intake status but might be linked to negative energy balance in cachexia. Expression of genes involved in serotonin signalling showed to be different between C26-TB mice and Lewis lung carcinoma-TB mice and was inversely associated with food intake. *In vitro*, using hypothalamic cell lines, serotonin repressed neuronal hypothalamic NPY secretion while not affecting messenger NPY expression, suggesting that serotonin signalling can interfere with NPY synthesis, transport, or secretion.

**Conclusions** Altered serotonin signalling is associated with changes in food intake behaviour in cachectic TB mice. Serotonin's inhibitory effect on food intake under cancer cachectic conditions is probably via affecting the NPY system. Therefore, serotonin regulation might be a therapeutic target to prevent the development of cancer-induced eating disorders.

**Keywords** Anorexia; Serotonin; Neuropeptide Y; Cancer; Cachexia

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\*Correspondence to: Klaske van Norren, Nutrition, Metabolism and Genomics Group, Division of Human Nutrition, Wageningen University, Bomenweg 2, 6703HD Wageningen, The Netherlands. Phone: +31-317-485790, Email: klaske.vannorren@wur.nl

## Introduction

Anorexia and consequently a reduced caloric intake<sup>1</sup> affects a majority of all cancer patients.<sup>2</sup> Cancer patients with good appetite and adequate caloric intake have higher survival rates than patients who suffer from anorexia and hence a

lower energy intake.<sup>3,4</sup> Cancer-induced anorexia is considered to be predominantly caused by failure of orexigenic and anorexigenic food intake-regulating systems in the hypothalamus to respond adequately to changes in energy balance.<sup>5</sup> This merits further elucidation of the specific mechanisms involved in this hypothalamic resistance to peripheral

neuroendocrine triggers. Several food intake-regulating systems have been mentioned to play a role in the onset of hypothalamic resistance, including the orexigenic triggers neuropeptide Y (NPY) and ghrelin, and anorexigenic melanocortin and CART systems. Cancer anorexia often develops during the progression of cachexia,<sup>6</sup> and the combined clinical picture is often called anorexia–cachexia syndrome.<sup>7</sup> Cachexia is a complex metabolic syndrome associated with underlying illness and is characterized by progressive loss of muscle (muscle wasting) with or without loss of fat mass. This results in weight loss, a reduced quality of life and a shortened survival time.<sup>6,8,9</sup> Although anorexia is often linked to cachexia and likely to be initiated by similar pathologies (tumour growth), it is unclear as to what extent anorexia and the metabolic alterations of cachexia affect each other, or to what extent these are distinct entities with their own pathology. Here, we use two tumour-bearing (TB) mouse models displaying similar development of cachexia. At the same time, they show opposite effects on food intake in response to tumour growth. This provides opportunities to disentangle processes involved in anorexia from those causing cachexia. By applying transcriptomics, we show that tumour-induced cachexia is associated with several distinct changes in food-intake, regulating mediators in the hypothalamus that appear to be independent of food intake status. Furthermore, we found alterations in hypothalamic serotonin signalling, and these changes were inversely associated with food intake. In addition, we used hypothalamic cell lines to explore the mechanisms involved in the interaction between serotonin and the NPYergic system.

## Materials and methods

### *Lewis lung tumour model (LLM)*

C57Bl/6 male mice (Harlan, Barcelona, Spain), weighing approximately 20 g, were placed on a standard *ad libitum* diet (Panlab, Barcelona, Spain) and had free access to water. Lewis Lung carcinoma (LLC) cells were obtained from exponential tumours. Under general anaesthesia,  $0.5 \times 10^6$  cells were injected intramuscularly in the hind leg. Four groups were included: C, control mice sacrificed at Day 14 ( $n=6$ ); TB-10, TB mice sacrificed at Day 10 ( $n=6$ ); TB-14, TB mice sacrificed at Day 14 ( $n=8$ ), and TB-17, TB mice sacrificed at Day 17 ( $n=6$ ). Body weight and food intake were measured daily. At the day of sacrifice, mice were anesthetized with a ketamine/xylazine mixture (i.p., Imalgene® and Rompun®, respectively), and blood was collected by cardiac puncture.

### *C26 colon adenocarcinoma tumour model*

Part of the data (Figure 1A) from this experimental arm with C26 TB mice have been published previously<sup>10</sup> but are briefly described again here for easy comparison with Lewis lung TB mice.

Male CDF1 (BALB/cx DBA/2) mice aged 6–7 weeks (Harlan Nederland, Horst, The Netherlands) were placed on a standard *ad libitum* diet (AIN93M, Research Diet Services, The Netherlands) and had free access to water.

Murine C26 adenocarcinoma cells were cultured and suspended as described previously.<sup>11</sup> Under general anaesthesia (isoflurane/N<sub>2</sub>O/O<sub>2</sub>),  $1 \times 10^6$  tumour cells in 0.2 mL Hank's balanced salt solution (HBSS) were inoculated subcutaneously into the right inguinal flank. Controls were sham-injected with 0.2 mL HBSS.

Two groups were included: C, control mice ( $n=6$ ) and TB ( $n=9$ ).

Body weight and food intake were measured three times a week. At Day 19, blood was collected by cardiac puncture under general anaesthesia.

In both tumour models, animals were individually housed 1 week before start of the experiment in a climate-controlled room (12:12 dark-light cycle,  $21^\circ\text{C} \pm 1^\circ\text{C}$ ).

After sacrifice, brain, hypothalamus, tumour, organs, and lower leg skeletal muscles (non-tumour leg) were weighted and frozen in liquid nitrogen and were stored at  $-80^\circ\text{C}$ . Carcass weight was calculated by body weight at day of section minus tumour weight.

All experimental procedures were made in accordance with the European community guidelines for the use of laboratory animals and complied with the principles of good laboratory animal care.

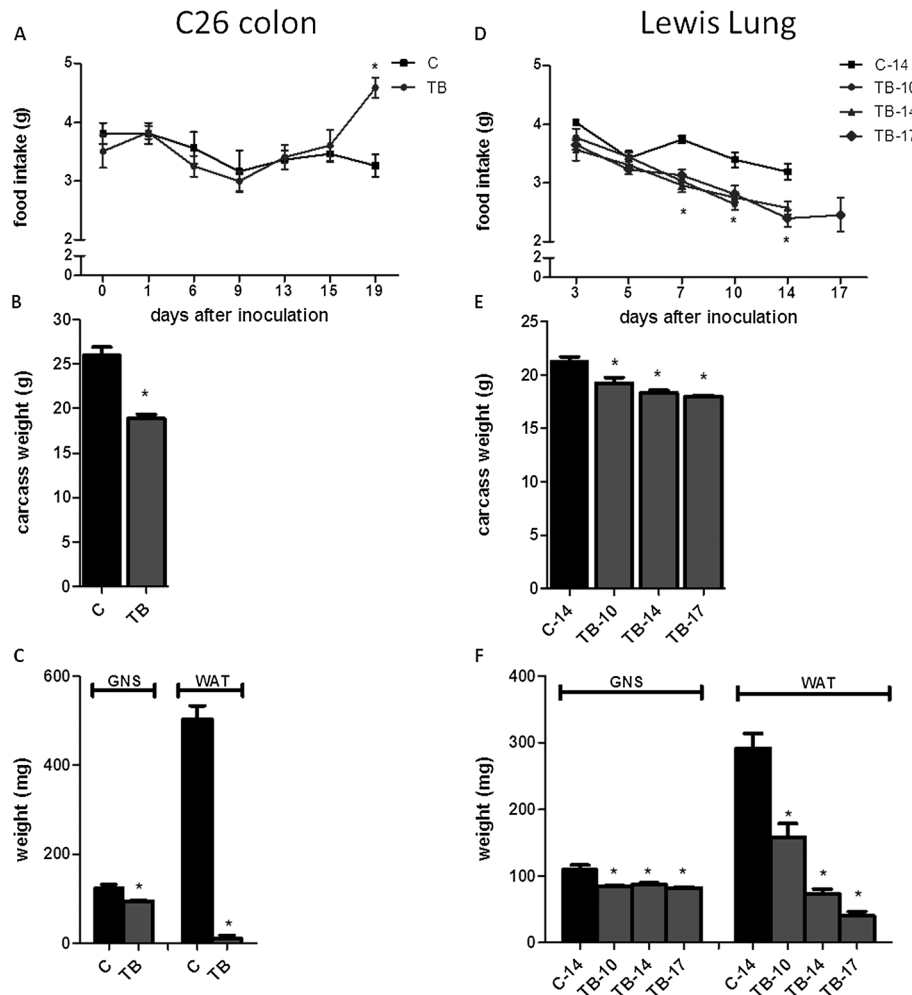
### *Cell culture and reagents*

Murine-derived hypothalamic neuronal cell lines hypoE-46 and hypo-A2/12 (CELLutions Biosystems Inc., Canada) were grown and maintained in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% heat-inactivated foetal calf serum, 100 unit/mL penicillin and 100 µg/mL streptomycin at  $37^\circ\text{C}$  under 5.0% CO<sub>2</sub>. Cells were grown in monolayers to 90% confluency. Then, medium was replaced by serum-free DMEM containing penicillin and streptomycin. After 4 h, cells were exposed to serotonin (100 µg/mL, 1 µg/mL, 10 ng/mL, or 100 pg/mL) for 24 h or 60 mM KCl for 15 min. After exposure, supernatant was collected and used for NPY measurements using an enzyme immunoassay (NPY EIA, Phoenix Pharmaceuticals, CA, USA). Cells were homogenized in 40 mM Tris, 1 mM ethylenediaminetetraacetic acid, 5 mM ethylene glycol tetraacetic acid, and 0.50% Triton X-100. Homogenates were used to measure protein content (Pierce Bicinchoninic acid Rockford, IL, USA). Serotonin cytotoxicity was determined by measuring lactate dehydrogenase leakage and effects on cell viability using a tetrazolium salt XTT assay (Roche Diagnostics, Mannheim, Germany).

### *Serotonin levels*

Hypothalamic samples were used for microarray experiments, while remaining brain parts were used to determine

**Figure 1** Effect of tumour inoculation on food intake, carcass weight and muscle, and fat weight in two cachectic models. (A) Time course of change in food intake in mice bearing the C26 tumour. (B) Carcass weight of C26 tumour-bearing mice at Day 19. (C) Weight of m. gastrocnemius and epididymal fat pads in C26 tumour-bearing mice at Day 19. (D) Time course of change in food intake of tumour-bearing mice bearing the Lewis lung carcinoma. (E) Carcass weight of Lewis lung carcinoma tumour-bearing mice at Days 10, 14, and 17. (F) Weight of m. gastrocnemius and epididymal fat pads in Lewis lung carcinoma tumour-bearing mice at Days 10, 14, and 17. \*Significantly different from C ( $P < 0.05$ ). Data are expressed as mean  $\pm$  SEM. C, sham-injected control; TB, C26-injected tumour bearing; C-14, sham-injected control sacrificed at Day 14; TB-10, Lewis lung carcinoma-injected tumour bearing sacrificed at Day 10; TB-14, Lewis lung carcinoma-injected tumour bearing sacrificed at Day 14; and TB-17, Lewis lung-injected tumour bearing sacrificed at Day 17. Carcass weight was calculated by body weight at day of section minus tumour weight.



serotonin levels. Brains were homogenized in 1 mL containing in 40 mM Tris, 1 mM ethylenediaminetetraacetic acid, 5 mM ethylene glycol tetraacetic acid, and 0.50% Triton X-100. Serotonin levels were measured using enzyme-immunoassay kits (BAE-5900, LDN, Nordhorn, Germany) and were corrected for the amount of protein (nanodrop spectrophotometer, Thermo Scientific).

### Statistics

Data were analysed by statistical analysis of variance followed by a *post hoc* Bonferroni test or by a Student's *t*-test.

Differences were considered significant at a two-tailed  $P < 0.05$ . Statistical analyses were performed using GraphPad Prism 5. For statistical analysis of microarray data, see microarray section.

### Microarray studies

Total RNA from the hypothalamus was isolated by using RNeasy lipid tissue kit (Qiagen, Venlo, The Netherlands). RNA concentrations were measured by absorbance at 260 nm (nanodrop). RNA quality was checked using the RNA 6000 n assay on the Agilent 2100 bioanalyser (Agilent

Techologies, Amsterdam, The Netherlands) according to the manufacturer's protocol. For each mouse, total RNA (100 ng) was labelled using the Ambion WT expression kit (Life Technologies, Bleiswijk, The Netherlands). Microarray experiments were performed by using affymetrix mouse gene ST 1.1.

From the C26 model experiment, four control samples and five samples from TB mice were included in this experiment; however, one control sample gave multiple spots on the array and was therefore excluded from analysis. The Lewis lung experiment included four controls, five TB-10, seven TB-14, and six TB-17 samples.

Array data were analysed using an in-house, online system.<sup>12</sup> Briefly, probesets were redefined according to Dai *et al.*<sup>13</sup> using remapped computable document format version 15.1 based on the Entrez gene database. In total, these arrays target 21 225 unique genes. Robust multi-array analysis was used to obtain expression values.<sup>14,15</sup> We only took genes into account that had an intensity >20 on at least two arrays, had an interquartile range throughout the samples of >0.1, and had at least seven probes per gene. Genes were considered differentially expressed at  $P < 0.05$  after intensity-based moderated *t*-statistics.<sup>16</sup> Further functional interpretation of the data was performed through the use of Ingenuity Pathway Analysis (IPA) (Ingenuity® Systems, www.ingenuity.com). Canonical pathway analysis identified the pathways from the IPA library of canonical pathways that were most significant to the data set. Genes from the data set that met the cut-off of 1.3-fold change and *P*-value cut off of 0.05 and were associated with a canonical pathway in the ingenuity knowledge base were considered for the analysis. To list up genes involved in food intake regulation, genes included in gene ontology (GO) 0002023, GO 0007631, GO 0008343, GO 0060259, and GO 0042755 (food intake, feeding behaviour, and eating behaviour) were analysed (gene ontology, tool for the unification of biology. The Gene Ontology Consortium (2000) *Nature Genet.* 25: 25–29). Also, genes pro-opiomelanocortin (ID: 18976), growth hormone (ID: 14599), Sst (ID: 20604), and pro-melanin-concentrating hormone (PMCh) (ID: 110312) were additionally looked at, since they also have been described to play a role in food intake behaviour.<sup>17</sup>

Array data have been submitted to the gene expression omnibus, accession numbers GSE44082 (C26 tumour) and GSE57190 (Lewis Lung tumour and hypothalamic cell lines). Data set containing the array data on hypothalamic tissues of C26 TB mice has been published previously.<sup>10</sup>

## Results

### *Body weight and food intake*

In both tumour models, TB mice showed a lower carcass weight, muscle weight, and white adipose fat mass (*Figure 1*)

compared with their healthy controls, reflecting that cachexia was present in mice bearing either the C26 or the Lewis Lung tumour.

Food intake changes in TB animals were found to differ between the two cachectic models. Mice bearing the C26 tumour was found to increase their food intake synchronously to loss of body weight,<sup>10</sup> while mice bearing the LLC showed a lowered food intake from Day 7 after tumour inoculation compared with controls (*Figure 1 A and D*). These opposing changes in food intake in TB mice in response to tumour growth of the two different tumours are used to disentangle shared mechanisms of cachexia from those important for food intake regulation.

### *Microarray analysis of the hypothalamus: comparison of C26 and LLM*

Expression of genes involved in food intake was analysed to determine if these could explain the opposing changes in food intake between C26 TB and LLC TB mice. A list of genes involved in food intake was generated by using GO categories involved in food intake regulation (M&M). Fold changes of expression of orexigenic and anorexigenic genes were compared between the two tumour models (*Figure 2A*). Interestingly, changes in gene expression of important mediators of food intake regulation, NPY, AgRP, and pro-opiomelanocortin showed to be similar in C26 TB mice and LLC TB mice compared with their controls even though food intake behaviour was different between the two models. Furthermore, gene expression of oxytocin showed to be altered in TB mice compared with controls and differed between the two models.

In addition, analysis of highly up-regulated genes (top 30 up-regulated genes in TB mice compared with controls) in both tumour models was performed. There was a strong overlap of genes that was highly up-regulated in both C26 TB and LLC TB mice compared with their controls, including NPY and AgRP. Furthermore, expression of inflammatory markers lipocalin 2, leucin-rich  $\alpha$ 2-glycoprotein 1, secretoglobulin 3a1, and oncostatin M receptor showed to be highly up-regulated in TB mice in both models. Taken together, this suggests that major alterations in hypothalamic mechanisms are similar for both tumour models.

### *Microarray analysis of the hypothalamus: serotonin signalling*

We hypothesized serotonin to play a crucial role in the opposing food intake behaviour between the C26 tumour and LLC tumour model. Therefore, expression levels of genes involved in serotonin signalling were compared between the two tumour models. C26 TB mice expressing compensatory eating behaviour in response to weight loss showed altered

**Figure 2** Heat map representation of gene expressions in the hypothalamus in C26 and Lewis lung carcinoma tumour-bearing mice. (A) Fold changes of orexigenic and anorexigenic genes relative to their control group were calculated and compared between C26 and Lewis lung carcinoma tumour-bearing mice. (B) Top 30 up-regulated genes in C26 tumour-bearing mice compared with their controls and top 30 up-regulated genes in Lewis lung carcinoma tumour-bearing mice compared with their controls. Twelve genes were overlapping between the C26 and Lewis lung carcinoma tumour model. Each row represents a gene, and each column represents a group of animals. Red colour indicates genes that were higher expressed as controls, and green colour indicates genes that were lower expressed as the controls. Black indicates genes whose expression was similar to compared with controls. ID: Entrez ID. C, sham-injected control; TB, C26-injected tumour-bearing; C-14, sham-injected control sacrificed at Day 14; TB-10, Lewis lung carcinoma-injected tumour bearing sacrificed at Day 10; TB-14, Lewis lung carcinoma-injected tumour bearing sacrificed at Day 14; and TB-17, Lewis lung carcinoma-injected tumour bearing sacrificed at Day 17.

A					B								
Gene	C26	LLM TB-10	LLM TB-14	LLM TB-17	ID	Description	Gene	C26	LLM day 10	LLM day14	LLM day 17	ID	Description
AgRP	1.6	1.5	1.8	2.3	11604	agouti related protein	Hbb-b1	3.8	2.7	5.0	7.4	15129	hemoglobin, beta adult major chain
Npy	1.9	1.5	2.1	2.6	109648	neuropeptide Y	Lcn2	7.2	4.7	4.9	3.6	16819	lipocalin 2
Npy1r					18166	neuropeptide Y receptor Y1	Alas2	2.7	2.1	4.1	5.4	11656	aminolevulinic acid synthase 2, erythroid
Npy2r					18167	neuropeptide Y receptor Y2	Slc4a1	1.7	2.0	3.2	4.2	20533	solute carrier family 4 (anion exchanger), member 1
Npy5r					18168	neuropeptide Y receptor Y5	Fam46c	1.9	1.8	3.2	4.2	74645	family with sequence similarity 46, member C
Ghr	1.2	1.2	1.3	1.4	208188	growth hormone secretagogue receptor	Rsd2	1.5	2.0	3.1	3.8	58185	radical S-adenosyl methionine domain containing 2
Gh	7.6		1.2	1.3	14599	growth hormone	Isg20	1.5	2.0	3.1	4.0	57444	interferon-stimulated protein
Ghr1					58991	ghrelin	Beta-s	2.6	1.8	3.0	3.6	100503605	hemoglobin subunit beta-1-like
Lep					16846	leptin	Acer2	1.7	2.6	2.9	3.2	230379	alkaline ceramidase 2
LepR		1.4	1.4	1.4	16847	leptin receptor	Vwf	1.9	2.2	2.8	2.9	22371	Von Willebrand factor homolog
Bsx	1.3	1.4	1.3	1.4	244813	brain specific homeobox	Ube2l6	1.4	1.6	2.6	3.4	56791	ubiquitin-conjugating enzyme E2L 6
Qrfp					227717	pyroglutamylated RFamide peptide	Gypa	1.3	1.5	2.4	2.6	14934	glycophorin A
Ace		1.2	1.2	1.2	11421	angiotensin I converting enzyme 1	Zbtb16	1.4	2.0	2.2	1.8	235320	zinc finger and BTB domain containing 16
Nkx2-1		1.2	1.2	1.2	21869	NK2 homeobox 1	Pgylrp1	1.2	1.5	2.1	1.9	21946	peptidoglycan recognition protein 1
Aoc3					11754	amine oxidase, copper containing 3	Npy	1.9	1.5	2.1	2.6	109648	neuropeptide Y
Ren1					19701	renin 1 structural	Plin4	2.4	1.3	2.0	1.5	57435	perilipin 4
Agtr	1.2				11606	angiotensinogen	H2-Aa		2.6	1.9	1.8	14960	histocompatibility 2, class II antigen A, alpha
Stat3					20848	signal transducer and activator of transcription 3	Hif3a	1.4	1.5	1.9	1.9	53417	hypoxia inducible factor 3, alpha subunit
Adrb3	1.2				11556	adrenergic receptor, beta 3	Il1r1	2.0	2.0	1.9	1.8	16177	interleukin 1 receptor, type I
Npw					381073	neuropeptide W	Ly6a	1.8	1.7	1.9	1.8	110454	lymphocyte antigen 6 complex, locus A
Ucn	1.2				22226	urocortin	Mgp	1.5	1.8	1.9	1.7	17313	matrix Gla protein
Pyy					217212	peptide YY	Cxcl9		2.4	1.8	1.6	17329	chemokine (C-X-C motif) ligand 9
P2ry1					18441	purinergic receptor P2Y, G-protein coupled 1	Il2rg		1.5	1.8	1.7	16186	interleukin 2 receptor, gamma chain
Pou4f1					18996	POU domain, class 4, transcription factor 1	Ch25h	1.5	1.8	1.8	2.0	12642	cholesterol 25-hydroxylase
Pmch					110312	pro-melanin-concentrating hormone	AgRP	1.6	1.5	1.8	2.3	11604	agouti related protein
Mchr1	1.2				207911	melanin-concentrating hormone receptor 1	Fn1		1.5	1.8	1.7	14268	fibronectin 1
Adm2					223780	adrenomedullin 2	Cd74		2.3	1.7	1.7	16149	CD74 antigen
Cartpt	1.2				27220	CART prepropeptide	A730020	1.2	1.7	1.7	1.6	100503044	RIKEN cDNA A730020M07 gene
Gigyl2					273331	GRB10 interacting GYF protein 2	Sult1a1	1.7	1.4	1.7	1.7	20887	sulfotransferase family 1A, member 1
Dmbx1	1.2				140477	diencephalon/mesencephalon homeobox 1	Lrg1	3.6	1.8	1.7	1.5	76905	leucine-rich alpha-2-glycoprotein 1
Helt		1.3			234219	helt bHLH transcription factor	Osmr	1.7	1.6	1.7	1.5	18414	oncostatin M receptor
a					50518	nonagouti	Phyhd1	1.9	1.3	1.6	1.7	227696	phytanoyl-CoA dioxygenase domain containing 1
Appl2					11804	amyloid beta (A4) precursor-like protein 2	Ppbb	3.2		1.4	1.8	57349	pro-platelet basic protein
Calca					12310	calcitonin/calcitonin-related polypeptide, alpha	Scgb3a1	5.8	1.7	1.4	1.3	68662	secretoglobulin, family 3A, member 1
Tcf15					21407	transcription factor 15	Agxt2l1	1.6	1.3	1.4	1.4	71760	alanine-glyoxylate aminotransferase 2-like 1
Nmu					56183	neuromedin U	Tekt4	1.7		1.3	1.7	71840	tektin 4
Nmur2	1.2				216749	neuromedin U receptor 2	Gh	7.6		1.2	1.3	14599	growth hormone
Ubr3					68795	ubiquitin protein ligase E3 component n-recognition 3	Prl	3.7		1.2	1.3	19109	Prolactin
Ace2					70008	angiotensin I converting enzyme 2	Gabrr2	1.7		1.3	1.3	14409	gamma-aminobutyric acid C receptor
Agtr1a	1.5				11607	angiotensin II receptor, type 1a	Stab2	1.8	1.7	1.3	1.3	192188	stabilin 2
Agtr1b					11608	angiotensin II receptor, type 1b	Mir181b	1.9		1.3	1.3	723890	microRNA 181b-1
Uchl1					22223	ubiquitin carboxy-terminal hydrolase L1	Cyp4f15	1.9		1.3	1.3	106648	cytochrome P450, family 4, polypeptide 15
App					11820	amyloid beta (A4) precursor protein	Mpz	1.8		1.3	1.3	17528	myelin protein zero
Grm7					108073	glutamate receptor, metabotropic 7	Mir379	2.3	1.7		1.3	723858	microRNA 379
Ntrk2					18212	neurotrophic tyrosine kinase, receptor, type 2	Olfr117	1.6			1.3	546770	olfactory receptor 1175, pseudogene
Tacr1					21336	tachykinin receptor 1	Mir411	3.0	1.3		1.3	723936	microRNA 411
Tacr3	1.3				21338	tachykinin receptor 3	Mir98	1.6	1.7		1.3	723947	microRNA 98
Adora2a	1.2				11540	adenosine A2a receptor	Tbr1	1.7	1.5	1.5	1.7	21375	T-box brain gene 1
Phf21a					192285	PHD finger protein 21A							
Chrb2	1.4	1.5			11444	cholinergic receptor beta polypeptide 2							
Cyp11b2					13072	cytochrome P450, family 11							
Gri1					14810	glutamate receptor NMDA1							
Ankrd26					232339	ankyrin repeat domain 26							
Hcrt		1.4	1.3	1.3	15171	hypocretin							
Hcrt2					387285	hypocretin (orexin) receptor 2							
Sst	1.2				20604	Somatostatin							
Uchl3					50933	ubiquitin carboxyl-terminal esterase L3							
Apin	1.2			1.2	623503	prolactin releasing hormone							
Prlhr	1.2				226278	prolactin releasing hormone receptor							
Prlh	1.2			1.2	72129	peroxisomal biogenesis factor 13							
Pex13					13134	dachshund 1							
Cntfr					12804	ciliary neurotrophic factor receptor							
Cnr1	1.2				12801	cannabinoid receptor 1 (brain)							
Bdnf					12064	brain derived neurotrophic factor							
Cpt1a					12894	carntine palmitoyltransferase 1a, liver							
Glp1r					14652	glucagon-like peptide 1 receptor							
Gri2b					14812	glutamate receptor NMDA2B							
Cck	1.2				12424	cholecystokinin							
Cckar		1.4		1.4	12425	cholecystokinin A receptor							
Crh	1.4	1.5			12918	corticotropin releasing hormone							
Crhr1					12921	corticotropin releasing hormone receptor 1							
Oxt	1.6	1.6	1.3	1.7	18429	oxytocin							
Oxtr					18430	oxytocin receptor							
Hrh3					99296	histamine receptor H3							
Gal					14419	galanin							
Trh	1.4	1.2			22044	thyrotropin releasing hormone							
Nmb					68039	neuromedin B							
Pomc		1.6	1.3	1.6	18976	pro-opiomelanocortin-alpha							
Mc4r	1.2	1.2		1.2	17202	melanocortin 4 receptor							

serotonin signalling and lower brain serotonin levels compared with their controls. In LLC TB mice, genes involved in serotonin signalling showed a different expression pattern compared with C26 TB mice. Expression of tryptophan

hydroxylase (*tph*) was strongly down-regulated in C26 TB mice compared with controls, while expression of this gene was not different from controls in LLC TB mice. Expression of *ddc* and *vmat*, genes involved in serotonin synthesis and



serotonin release, respectively, showed to be down-regulated in C26 TB mice, whereas LLC TB mice showed higher expression of these genes (Figure 3B). Serotonin brain levels in LLC TB mice showed to be slightly elevated at Day 10, just after the onset of anorexia, while levels were lower in C26 TB mice compared with controls (Figure 3C).

### *Hypothalamic cell lines: effect of serotonin on messenger neuropeptide Y and neuropeptide Y secretion*

Murine-derived hypothalamic cell lines hypoE-46 and hypoA-2/12 were 24 h exposed to serotonin (100 pg/mL–100 µg/mL). Serotonin showed to decrease NPY secretion in the hypoE-46 cells, while it did not have any effect on messenger NPY (Figure 4). In hypoA-2/12 cells, serotonin did not have an effect on either NPY secretion or NPY gene expression. Basal expression level of serotonin receptors between the two cell lines showed to be significantly different for receptors 5HT1b, 5HT1d, 5HT2a, and 5HT2b (Table 1). The effect of serotonin on gene expression of 5HT receptors was not different between the two cell lines, except for 5HT2b, which was down-regulated in hypoE-46 cells and up-regulated in hypoA-2/12 cells after serotonin exposure.

## Discussion

Cancer anorexia has been explained by an inability of the hypothalamus to respond adequately to triggers from the periphery during a situation of negative energy balance.<sup>18</sup> Here, we show that this impaired response of food-intake control is associated with altered serotonin signalling and probably stunted NPY post-transcriptional regulation in the hypothalamus. In addition, this serotonin regulation and brain serotonin levels were inversely associated with food intake. *In vitro*, serotonin was found to modulate NPY release by hypothalamic neurons. Taken together, this suggests that serotonin is likely to play a crucial role in the failure of the NPYergic system during cancer anorexia.

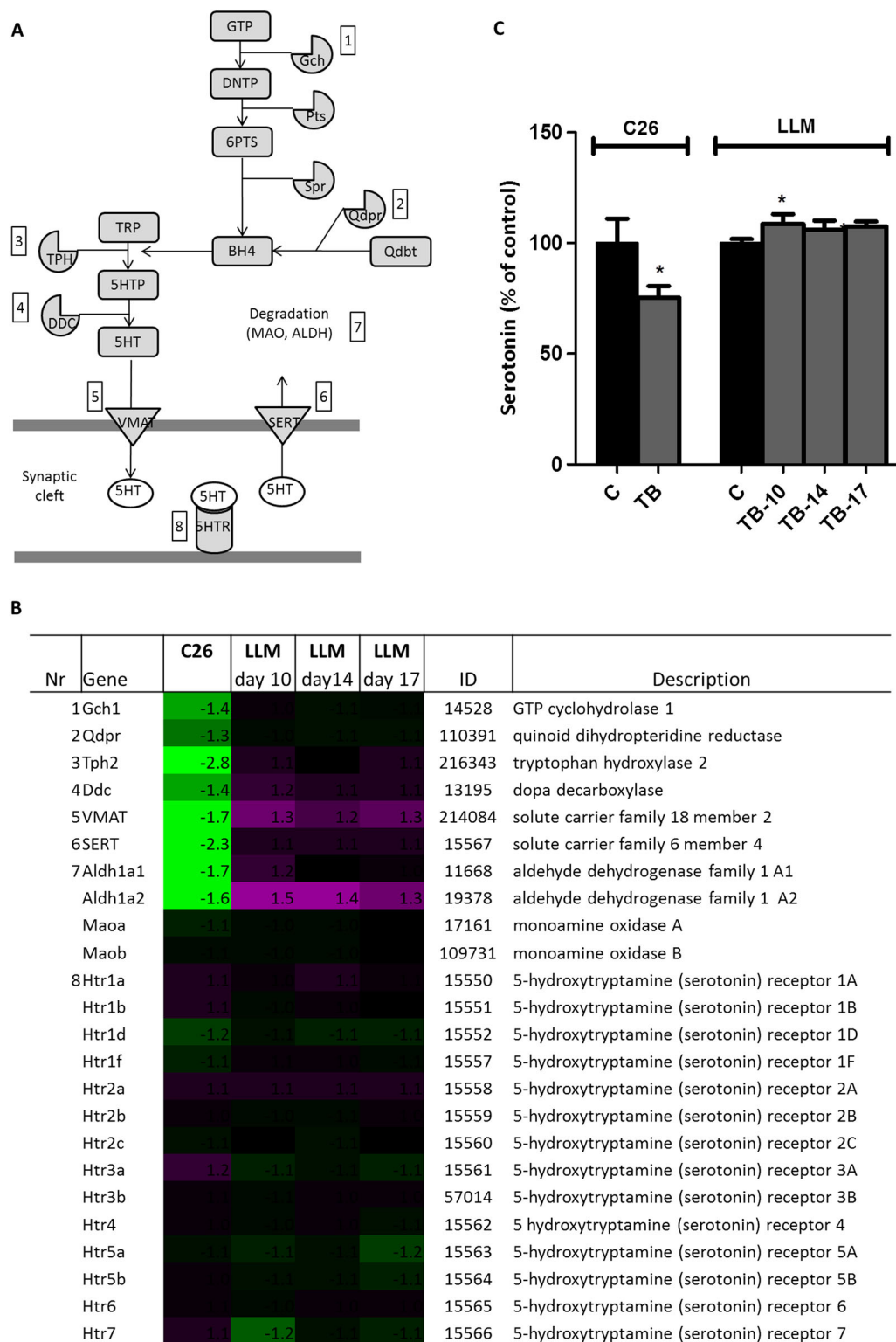
By comparing two cancer models sharing common characteristics of tumour-induced muscle wasting and fat loss while displaying opposite effects on food intake behaviour, we are able to disentangle anorexia from cachectic processes. Peripheral markers for cachexia reflecting weight loss, muscle loss, and fat loss were comparable between the two tumour models. In addition, the most prominently (top 30-based) induced hypothalamic genes compared with healthy controls showed to be similar between the two models. This suggests that there is a common subset of genes that is strongly induced during tumour growth and progression of cachexia, despite the differences in type of tumour, inoculation site,

strain of mice, and independent from effects on food intake behaviour. These changes might represent markers for cachexia or tumour-induced inflammation as gene expression of strongly up-regulated inflammatory genes was overlapping between the two models.

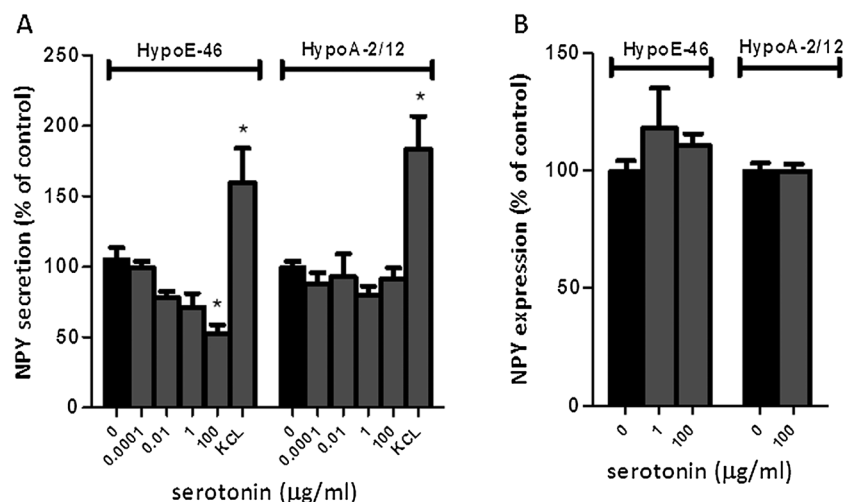
Interestingly, also a majority of genes involved in food intake regulation showed to be similar between the models, even though ultimate effects on food intake were different for the two models. Food intake and drive to eat are the outcomes of numerous metabolic, physiological, and behavioural cues including satiety, hunger and reward, and learning. Therefore, changes on individual genes are difficult to interpret, as the impact on food intake is not similar between these single genes. Furthermore, it might be that, considering the complexity of food intake regulation, by using GO classification on food intake behaviour, some genes are ignored that might be important as well. Finally, a limitation of the current set-up is that we study expression in the hypothalamus only. Although the hypothalamus is a crucial site of food intake regulation, not all processes involved in food intake are regulated in the hypothalamus.<sup>19</sup> Gene expression of NPY, one of the most potent food stimulating neuropeptides in the hypothalamus,<sup>20</sup> showed to increase in both C26 TB and LLC TB mice and was among the strongest induced genes in both models. This suggests that expression of NPY in cachectic TB mice is independent of food intake status. NPY is considered to act as a sensor and regulator of energy balance by altering food intake in energy deficits, which is supported by findings that NPY is stimulated in conditions of negative energy balance like food restriction,<sup>21</sup> food deprivation,<sup>22,23</sup> and exercise.<sup>24,25</sup> Several studies have shown that in cachectic TB rodents, NPY messenger RNA (mRNA) in the hypothalamus is elevated.<sup>26–29</sup> However, this elevation of mRNA NPY does not reflect food intake behaviour<sup>27,29</sup> and does not correspond to NPY levels in the hypothalamus.<sup>27</sup> In contrast to these elevated messenger NPY levels, anorectic TB rodents have lower paraventricular nucleus (PVN) hypothalamic NPY levels<sup>30,31</sup> and lower NPY immunostaining of fibres innervating various hypothalamic nuclei.<sup>32</sup> On the other hand, mice bearing an A375 tumour that do not suffer from cachexia and weight loss did not show this elevation of NPY mRNA,<sup>26</sup> suggesting that cachexia is an important trigger in stimulation of NPY mRNA. This might explain that in both the C26 model and LLC model, messenger NPY was highly up-regulated in TB mice compared with controls, despite their difference in food intake behaviour. Consequently, we hypothesize that failure of the NPYergic system taking place at a post-transcriptional level, for example, by impaired translation, synthesis, transport, or secretion, plays a role in the development of tumour-induced anorexia.

In this study, we also found serotonin signalling to be differently regulated between two types of tumour-induced cachexia models, in addition to their opposed effects on food intake behaviour. In C26 TB mice, serotonin signalling was

**Figure 3** Serotonin signalling in C26 and Lewis lung carcinoma tumour-bearing mice. (A) Schematic view of genes involved in serotonin signalling. (B) Heat map of fold changes of expressions of genes involved in serotonin signalling. (C) Serotonin level in brain relative to control mice in C26 and Lewis lung carcinoma tumour-bearing mice. Values are expressed as mean  $\pm$  SEM. C, sham-injected control; tumour bearing, injected with  $1 \times 10^6$  tumour cells; DNTP, 7,8-dihydroneopterin triphosphate; 6PTS, 6-pyruvoyl-tetrahydropterin; q-dbt, q-dihydrobiopterin; TRP, tryptophan; 5HT, 5-hydroxytryptamine (serotonin); MAO, monoamine oxidase; and ALDH, aldehyde dehydrogenase.



**Figure 4** Effect of serotonin on messenger neuropeptide Y and neuropeptide Y secretion in hypothalamic cells. Murine-derived hypothalamic cell lines were 24 h exposed to various concentrations serotonin. Potassium chloride was used to depolarize cells (positive control). (A) Effect of serotonin on NPY secretion in hypoE-46 and hypoA-2/12 cells. (B) Effect of serotonin on neuropeptide Y gene expression in hypoE-46 and hypoA-2/12 cells. \*Significantly different from C ( $P < 0.05$ ). Data are expressed as mean  $\pm$  SEM.



**Table 1** Effect of serotonin on gene expression in hypothalamic cells

Gene	HypoE-46		HypoA-2/12		ID	Description
	Control	100 µg 5HT	Control	100 µg 5HT		
Htr1a	12.0	12.5	11.8	12.7	15 550	5-Hydroxytryptamine receptor 1A
Htr1b	19.0	17.6	41.8 <sup>b</sup>	31.9	15 551	5-Hydroxytryptamine receptor 1B
Htr1d	20.2	13.0 <sup>a</sup>	15.8 <sup>b</sup>	14.5	15 552	5-Hydroxytryptamine receptor 1D
Htr1f	17.3	15.7	16.7	15.9	15 557	5-Hydroxytryptamine receptor 1F
Htr2a	32.1	24.4 <sup>a</sup>	104.0 <sup>b</sup>	213.7 <sup>c</sup>	15 558	5-Hydroxytryptamine receptor 2A
Htr2b	18.6	16.2	12.9 <sup>b</sup>	16.4	15 559	5-Hydroxytryptamine receptor 2B
Htr2c	9.3	9.5	9.0	10.3	15 560	5-Hydroxytryptamine receptor 2C
Htr3a	10.2	9.5	9.6	10.5	15 561	5-Hydroxytryptamine receptor 3A
Htr3b	12.1	13.1	12.1	11.9	57 014	5-Hydroxytryptamine receptor 3B
Htr4	20.9	19.9	19.0	19.2	15 562	5-Hydroxytryptamine receptor 4
Htr5a	13.9	14.8	13.5	14.0	15 563	5-Hydroxytryptamine receptor 5A
Htr5b	21.6	18.3	18.5	17.7	15 564	5-Hydroxytryptamine receptor 5B
Htr6	21.6	19.4	21.9	19.8	15 565	5-Hydroxytryptamine receptor 6
Htr7	17.9	17.8	13.9	14.7	15 566	5-Hydroxytryptamine receptor 7

Murine-derived hypothalamic cell lines hypoE-46 were 24 h exposed to various concentrations serotonin. Gene expression of serotonin receptors (5HTRs) were determined by using microarrays.

<sup>a</sup>Significantly different from control in hypoE-46 cells.

<sup>b</sup>Significantly different from hypoE-46 cells basal expression.

<sup>c</sup>Significantly different from control in hypoA-2/12 cells ( $P > 0.01$ ).

strongly down-regulated and inversely associated with an increased food intake. Anorectic Lewis lung TB mice displayed less pronounced changes in transcript levels of gene involved in serotonin signalling compared with C26 TB mice. Moreover, transcriptional regulation was different from mice-bearing C26 tumour. Furthermore, brain serotonin levels were significantly elevated in TB mice at Day 10 compared with controls, shortly after the manifestation of anorexia. This effect was less clear at Days 14 and 17, suggesting that effects on serotonin are more prominent during the initial stages of anorexia development. It has been postulated that cancer anorexia

develops by waves of brain activation,<sup>33</sup> each of them not necessarily mediated by the same mediators. It could be that serotonin might play a role in the initiation of anorexia. Hypothalamic serotonin is an important mediator in the regulation of satiety and hunger.<sup>34</sup> In cancer-induced anorexia, serotonin is considered a key player in the induction of anorexia.<sup>35,36</sup> In rodents, tumour-driven elevation of hypothalamic serotonin has been associated with reduced food intake.<sup>31,37,38</sup> Furthermore, this elevation of serotonin was not present in the paired control group, meaning that reduced food intake itself does not result in an elevation of hypothalamic serotonin. In



humans, a practical and non-invasive method to measure site-specific changes on serotonergic activity in the hypothalamus is not yet available, but increased levels of tryptophan, the precursor of serotonin, have been measured in cerebral spinal fluid of cancer anorectic patients.<sup>39</sup>

The exact mechanism on how serotonin is able to regulate food intake is not yet understood. A cross interaction of serotonin with NPYergic system has been discussed.<sup>31</sup> Serotonin signalling is negatively associated with NPY activity.<sup>40–42</sup> Active lowering of serotonin levels results in increased NPY levels,<sup>43</sup> while induction of 5-HT signalling showed to reduce NPY levels in rat hypothalamus.<sup>44</sup> More importantly, serotonin is able to repress food intake, despite weight loss-induced elevation of messenger NPY,<sup>45</sup> suggesting that serotonin might act as an interfering factor between transcription and NPY secretion. Therefore, we hypothesized that serotonin might interfere with NPY signalling at a post-transcriptional level. In this way, it could play a role in post-transcriptional failure of the NPY system during certain forms of cancer.

To determine serotonin's ability to repress NPY secretion, we used two murine-derived hypothalamic cell lines. We showed that serotonin is able to reduce NPY secretion in hypoE-46 cells, a cell line suggested to originate from the PVN,<sup>46</sup> without affecting NPY gene expression, supporting the hypothesis that serotonin acts post transcriptionally.

Serotonin did not have an effect on NPY secretion in hypoA-2/12 cells, a cell line suggested to originate from the arcuate nucleus (ARC) or supraoptic nucleus.<sup>47</sup> In lean and obese Zucker rats, increased levels of serotonin, induced by chronic administration of fluoxetine, resulted in reduced food intake and reduced levels of NPY in the PVN, but not in the ARC and other areas of the hypothalamus.<sup>40</sup> This suggests that NPY release by PVN neurons is more sensitive to serotonin and might explain the difference in response to serotonin between the two cell lines.

The two cell lines did not show differences in their general response to serotonin as reflected by expression of genes involved in serotonin signalling. An exception to this was the effect of serotonin on expression of the 5HT2a receptor, which was down-regulated in response to serotonin in the hypoE-46 and up-regulated in the hypoA-2/12 cell line. Moreover, basal expression of 5HT1b and 5HT2a, and 5HT2b and 5HT2c differed between the two cell lines. In the ARC nucleus,

serotonin has been reported to inhibit NPY release by hyperpolarization of AgRP/NPY neurons via 5HT1B receptors.<sup>48</sup> The role of 5HT2 receptors might be a target for further research on their role in NPY regulation. However, as NPY secretion is via granule exocytosis,<sup>49</sup> also, a receptor-independent process like serotonylation of guanosine triphosphatases (GTPases) might be implicated. Serotonylation is a process, where intracellular serotonin is able to activate small GTPases via transamination. Dependent on the type of GTPases that are activated, several processes dependent on granule exocytosis can be affected, which has been reported for insulin secretion by pancreatic  $\beta$  cells<sup>50</sup> or platelet aggregation.<sup>51</sup>

In this study, we provide evidence that cancer anorexia might be due to post-transcriptional failure of the NPYergic system in the hypothalamus to respond to increased energy requirement in cancer cachexia. We show that serotonin is able to affect NPY secretion *in vitro*. In addition, serotonin signalling is found to be altered in TB mice, suggesting that serotonin might play a crucial role in failure of hypothalamic NPY signalling and subsequently the development or sustainment of cancer anorexia.

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## Conflict of interest

J. Dwarkasing, M. Boekschoten, J. Argilès, S. Busquets, F. Penna, M. Toledo, A. Laviano, and R. Witkamp have nothing to declare. M. van Dijk is an employee of Nutricia Research, a medical nutrition company. K. van Norren is a guest employee of Nutricia Research, a medical nutrition company.

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