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Epidemiological support for a multifactorial aetiology of Kashin-Beck disease in Tibet

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Abstract We carried out a cross-sectional study in 12 rural villages in order to identify the risk factors for Kashin-Beck disease in Tibet. Children aged 5–15 years (*n*=575) were examined and their corresponding houses were visited. Samples were collected in order to study fungal contamination of stored grain and the organic matter content of drinking water. Multivariate analysis was performed using logistic regression and population attributable fractions were computed to estimate the impact of each factor. The following variables were independently associated with the disease: age, gender, low socio-economic status, indicators of a poorly diversified diet, iodine deficiency and small water container size (with higher organic matter levels in small containers). Selenium deficiency was severe in all study subjects. The degree of fungal contamination of barley grain was related to the highest percentage of cases (65%) in a sample of the study population. Higher urinary iodine levels were not associated with decreasing prevalence rates when *Alternaria* sp. was isolated. The data that we report supports the hypothesis that Kashin-Beck disease occurs as a consequence of oxidative damage to cartilage and bone cells when associated with decreased antioxidant defence. Another mechanism that may coexist is bone remodelling stimulated by thyroid hormones whose actions can be blocked by certain mycotoxins.

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Résumé Nous avons réalisé une enquête transversale dans 12 villages ruraux afin d'identifier les facteurs de risque de la maladie de Kashin-Beck au Tibet. Les enfants âgés de 5 à 15 ans (*n*=575) et leurs maisons ont été examinés. La contamination fongique des céréales et le contenu en matières organiques de l'eau de boisson ont été étudiés dans des échantillons de la même population. L'analyse multivariée a été effectuée par régression logistique et les fractions attribuables ont été calculées afin d'estimer l'impact de chaque facteur. Les variables suivantes étaient indépendamment associées à la maladie: l'âge, le sexe, un bas état socio-économique, une alimentation peu diversifiée, la déficience en iode et une petite taille du réservoir à eau (présentant un contenu en matières organiques plus élevé). La déficience en sélénium était sévère chez tous les enfants de l'étude. Le degré de contamination fongique des grains d'orge expliquait le plus haut pourcentage de cas (65%) dans le sous-modèle mycologique. L'iode urinaire n'était pas associé à la maladie lorsque *Alternaria* sp. était isolé. Les résultats sont compatibles avec des dommages oxydatifs aux cellules osseuses en présence d'une défense antioxidante déficiente. Un autre mécanisme co-existant possible est le remodelage osseux de l'os stimulé par les hormones thyroidiennes qui peut être bloqué par certaines mycotoxines.

Introduction

The aetiology of Kashin-Beck disease (KBD) remains controversial. Recent scientific literature is dominated by three hypotheses based respectively on selenium deficiency, mycotoxins from contaminated storage grains, and organic matter (fulvic acid, FA) in drinking water. A Medline search from 1968 to 1998 yielded 124 relevant papers of which 61 discussed the aetiology, based either on original studies or from a review of existing evidence. From 1990 to 1998, selenium deficiency represented 55% of the discussed hypotheses, FA 26% and mycotoxins 17%. Other factors such as heavy metals and heredi-

tary factors were mainly discussed in articles published before 1990. In several recent papers, the predominant factors were combined in multifactorial aetiological models, based on the concept of oxidative stress. Peng et al. [9, 10] suggested that selenium deficiency may be considered as the underlying factor responsible for a defective antioxidant defence system, thus predisposing cartilage cells to lipid peroxidation initiated by free radical carriers, FA and mycotoxins. Mycotoxins are indeed known to induce lipid peroxidation, an effect that can be prevented by antioxidants [5, 11, 12]. In animal experiments, FA and selenium deficiency have been reported to be associated with degeneration of articular cartilage, modification of collagen I in bone and collagen II in cartilage, development of fibrocartilage at the articular surface and impaired formation of subchondral bone [14]. The object of this study was to identify the risk factors for KBD in Tibet and to estimate the relative importance of each factor. The study brings together a series of factors measured in the same study population, some of which have been discussed in detail in other papers [2, 3, 4, 8].

Materials and methods

In May 1995, a cross-sectional survey was carried out in 12 rural villages located in four counties of Lhasa prefecture. The distance to urban areas, level of socio-economic development and of agricultural activity were kept homogeneous based on qualitative information obtained at the county level.

All children aged 5–15 years were invited to the village centre for clinical examination. Parents were asked to bring children who were absent at the time of our visit, and those children who were at school at the time were collected by car when this was feasible. From May 22 to June 8 1995, a total of 575 out of a possible 685 (84%) children were recruited from 322 families. Blood and urine samples were obtained and iodine and selenium status were assessed. Methods used for clinical chemistry measurements have been reported elsewhere [8].

The clinical examination with regard to KBD was carried out by a physical therapist with considerable clinical experience in this disease. Kashin-Beck disease (KBD) was diagnosed when a child living in an endemic area of KBD presented with joint pain and joint deformity, or with restricted joint motion without any history of trauma or local inflammation [7]. A family was considered as affected by KBD if at least one of the examined children had the disease.

On the same day as the clinical examination the children's homes were visited and a family questionnaire and standardised house observation sheet were used to collect and record data. Both data collection forms had been pre-tested and modified before the start of the survey.

The family questionnaire was completed during a face-to-face interview by one of four trained non-medical Tibetan interviewers. The family members interviewed were the child's mother (48.5%), the child's father (33.2%), a grandparent (11.7%) or another family member (6.6%). The socio-economic status of the family was assessed by considering the reported annual income, the parents' occupation and the parents' ability to read Tibetan (proxy variable for education). The availability of food in the household at the time of the visit was assessed by using a questionnaire listing a predefined group of food items. Quantities were recorded in local units. The child's main daily activities and hygiene practices were also recorded.

Structured observations were carried out by a team of three investigators. The type of storage containers for drinking water and grain and the characteristics of the grain storage room were recorded. Ventilation of the storage room was estimated by counting the number of apertures (doors, windows). Grain samples were taken and grain humidity measured on site using a SAMAP-o-meter. In October 1995 (post-harvest), new grain humidity measurements were made, and samples were collected from a sub-sample of the same families. Fungal contamination of barley grain was studied in a sample of 60 families which included 126 children already in the study. In order to increase the degree of contrast in this analysis, affected families were selected according to the presence of several (≥2) children with KBD while unaffected families were those with several children without the disease. Methods for mycological analysis have been reported elsewhere [3]. Water samples were also obtained from a sample of the study population (n=69 families) and analysed for total organic carbon (TOC) levels.

Statistical analysis. Data were analysed using Stata 6.0 software. Multiple logistic regression was used to estimate the independent effect of each factor. Age- and sex-adjusted analysis was carried out and adjusted odds ratios (ORs) with 95% confidence limits were computed. For variables measured at the family level, standard errors were adjusted for clustering on each family. Means for continuous variables were compared using either one-way analysis of variance or the Kruskall-Wallis test as appropriate. Variables with P-values of less than 0.05 were entered in a multivariate model and removed using a backward stepwise method. Missing categories were included in order to evaluate bias due to missing information. Adjusted population attributable fractions (AF) were calculated in order to appreciate the proportion of cases attributable to each factor. Variables were recoded so that higher risk categories were compared to the lowest risk category. The area under ROC (receiver operator characteristics) curve and the percentage of correctly classified subjects (at maximum specificity + sensitivity) were used to assess the predictive power of the model.

Results

Among the 575 children, 280 were diagnosed clinically as having KBD (48.7%). The joints most often affected were the ankles (81%), the knees (56%) and the elbows (56%). Joint deformity was recorded in 266 children (95%), pain in 163 (58%) and limitation of joint motion in 60 (21%). The prevalence rate per village varied from 0% to 100% with a median of 60.6% (interquartile range 16.5–73.1%). In 181 out of 322 families (56%), at least one child had KBD.

The proportion of KBD children increased from 34% in the 5- to 7-year olds to 61% in the 12- to 15-year olds (Table 1). In all age groups, boys were more often affected than girls. Given the strong association of KBD with age and gender all further analyses were adjusted for these potentially confounding variables. KBD was not associated with family size, but patients with KBD were more likely to have a sibling who also had the disease than unaffected children.

Selenium and iodine status

Overall selenium deficiency was severe (38% with serum selenium concentrations <5 ng/ml), but selenium levels did not vary significantly between cases and healthy subjects.

Table 1 Factors associated significantly with Kashin-Beck disease in 12 rural villages in Tibet. Age- and sex-adjusted analysis

Factor	Healthy	KBD	(% KBD)	OR[a]	(95% CI)	*P*-value[b]
Total study population	295	280	(48.7)			
Demographic characteristics						
Gender (*n*=575)						
Female	163	107	(39.6)	1.0	–	
Male	132	173	(56.7)	2.0	(1.4–2.8)	<0.001
Age (*n*=575)						
5–7 years	120	62	(34.1)	1.0	–	*<0.001*
8–11 years	102	103	(50.2)	2.0	(1.3–2.9)	
12–15 years	73	115	(61.2)	3.0	(2.0–4.7)	
Other KBD case in family (*n*=344)[c]						
No	88	40	(31.3)	1.0	–	
Yes	72	144	(66.7)	5.6	(2.7–11.6)	<0.001
Iodine status						
Urinary iodine (µg/dl; *n*=557)						
<1.0	62	98	(61.3)	1.0	–	*<0.001*
1.0–1.9	104	101	(49.3)	0.7	(0.4–1.1)	
≥2.0	118	74	(38.5)	0.4	(0.3–0.7)	
Serum TSH (mU/l; *n*=536)						
<5 mU/l	156	113	(42.0)	1.0	–	*<0.001*
5–9.9 mU/l	71	88	(55.3)	1.8	(1.2–2.7)	
≥10 mU/l	47	61	(56.5)	2.2	(1.4–3.6)	
Serum T4 (µg/dl; *n*=532)						
<6.0	63	79	(55.6)	1.0	–	*0.002*
6–8.9	113	119	(51.3)	0.8	(0.5–1.2)	
≥9	96	62	(39.2)	0.5	(0.3–0.8)	
Serum TBG (mg/l; *n*=544)						
<17.0	45	91	(66.9)	1.0	–	*<0.001*
17–19.9	95	85	(47.2)	0.4	(0.3–0.7)	
≥20	136	92	(40.4)	0.4	(0.2–0.6)	
Socio-economic characteristics						
Occupation of parents (*n*=565)						
Farmer	155	204	(56.8)	1.0	–	
Nomad/seminomad	78	39	(33.3)	0.4	(0.2–0.7)	<0.001
Secondary-tertiary	56	33	(37.1)	0.5	(0.2–0.9)	0.019
Family income (*n*=532)						
≥500 Y	97	46	(32.2)	1.0	–	*<0.001*
100–499 Y	81	98	(54.7)	2.6	(1.4–4.8)	
<100 Y	94	116	(55.2)	2.7	(1.5–4.8)	
Child's practices						
Activities (*n*=526)						
Attends school	167	143	(46.1)	0.4	(0.2–0.6)	<0.001
Looks after domestic animals	44	73	(62.4)	1.9	(1.2–2.9)	0.006
Personal hygiene (*n*=524)						
Never washes	145	174	(54.5)	1.0	–	*<0.001*
Washes without soap	26	24	(48.0)	0.7	(0.3–1.2)	
Washes with soap	96	59	(38.1)	0.5	(0.3–0.8)	
Food availability in household (*n*=538)						
Presence of basic food items (barley, wheat, tsampa)						
3 items	86	51	(37.2)	1.0	–	*<0.001*
2 items	160	149	(48.2)	1.7	(1.0–2.9)	
1 item	26	52	(66.7)	3.8	(1.8–7.7)	
0 items	4	10	(71.4)	4.2	(0.8–22.4)	
Quantity of grains (if present, in kh[d])						
Barley	20 (9–35)	10 (5–20)				0.018
Wheat	8 (4–29)	4 (2–12)				0.042
Tsampa flour origin						
From other village	87	53	(37.9)	1.0	–	
Only own production/none	189	209	(52.5)	1.8	(1.1–3.1)	0.022

Table 1 (continued)

Factor	Healthy	KBD	(% KBD)	OR[a]	(95% CI)	*P*-value[b]
Other food products						
Colza	85	101	(54.3)	1.6	(1.0–2.6)	0.049
Peas	8	1	(11.1)	0.2	(0.0–0.7)	0.019
Water storage (*n*=520)						
Water storage container						
Big	159	113	(41.5)	1.0	–	*<0.001*
Medium	66	68	(50.7)	1.5	(0.9–2.7)	
Small	38	76	(66.7)	3.0	(1.6–5.8)	
Grain storage						
Type of storage room (*n*=513)						
Separate room/living/other	249	238	(48.9)	1.0	–	
Tent	5	21	(80.8)	5.5	(1.5–19.8)	0.009
Type of floor (*n*=488)						
Mud/mud-stone	202	232	(53.5)	1.0	–	
Concrete	36	18	(33.3)	0.5	(0.2–1.0)	0.047
Barley humidity % in October (new grains; *n*=240)						
Lower tertiles (<19.2%)	91	63	(40.9)	1.0	–	
Highest tertile (≥19.2%)	37	49	(57.0)	2.1	(1.0–4.5)	0.045
Mycological analyses						
Mycological index (ATD; *n*=126)[e]						
0 fungi	44	8	(15.4)	1.0	–	*<0.001*
1 fungus	28	20	(41.7)	5.6	(1.3–24.1)	
2–3 fungi	3	23	(88.5)	79.3	(12–524)	

[a] Odds ratio, adjusted for age and sex with 95% confidence interval
[b] *P*-values in italics are *P*-values for trend for ordinal variables
[c] (If ≥ 2 children) in "mixed" villages
[d] 1 Khai=20 kg; figures represent median and interquartile range
[e] Mycological index for presence of *Alternaria* sp., *Trichothecium roseum* and *Drechslera* sp.

Cases were more likely to be iodine deficient than those without the disease. Urinary iodine, serum thyroxine (T4) and serum thyroxine-binding globulin (TBG) were significantly lower in KBD patients while serum thyrotropin (TSH) was significantly higher.

Socio-economic characteristics

Children from families where at least one parent was noted to have nomad activities (21%) were less affected than children from farmer parents. Children from parents with a secondary (e.g. carpenter, road worker, driver) or tertiary (e.g. teacher, village leader, commercial activities) occupation (together 16%) also had a lower risk of developing the disease.

Illiteracy of both (39%) or of one of the parents (48%) was not associated with KBD. Children from families with an annual income of 500 Yuan or more were significantly less affected than those from lower income families. Reported family income was correlated (at $P<0.01$ level) with, among other parameters, a secondary or tertiary occupation and the quantity of most food items.

Children who attended school had significantly less KBD than children of the same age who did not. Inversely, children who looked after domestic animals were more affected. Other activities (field work, house work, playing) were not associated with KBD.

With regard to hygiene, parents were asked when the child had last washed. Up to 55% of the children were reported never to wash (healthy children 49%, KBD cases 62%). Among those who did wash, the median 'delay' was 6 weeks, ranging from 0 (same week) to 40 weeks (previous summer), 76% of them used soap for washing.

Food availability

The main 'storage food' in the studied population was barley and wheat. Of the total quantity of grain sown or planted during the relevant year in the study villages, 55% was barley, 20% wheat, 14% colza, 7% peas, 2% potatoes and radishes, and 1% peas. At the time of the survey, barley was present in 82% of the visited families, wheat in 34%, and wheat or barley flour ("tsampa") in 91%. The number of different basic food items in the family home was associated with the occurrence of KBD. The quantity of barley and wheat, when present, was also higher in unaffected families, although the quantity of flour was not. Flour from other villages was associated inversely with KBD. Other food products present at the time of the survey were salt (present in 96% of the families), black tea (78%), butter (39%), colza (41%), beans (9%), rice (9%), dried meat (4%), potatoes (3%) and peas (2%). Except for colza and peas, these items were not significantly associated with KBD.

Table 2 Risk factors of Kashin-Beck disease in Tibet: multivariate analysis (logistic regression) and adjusted population attributable fractions

Variable	Adj. OR[a]	(95% CI)	*P*-value[b]	AF% (SE)[c]
Gender				
Female	1.0	–		
Male	2.4	(1.6–3.6)	<0.001	17.5 (3.9)
Age				
5–7 years	1.0	–	*<0.001*	
8–11 years	2.1	(1.3–3.5)		10.6 (3.4)
12–15 years	4.2	(2.5–7.0)		17.9 (3.1)
Urinary iodine (µg/dl)				
≥2.0	1.0	–	*<0.001*	
1.0–1.9	1.6	(1.0–2.5)		6.3 (3.1)
<1.0	2.3	(1.4–3.7)		9.0 (2.7)
Family income				
≥500 Y	1.0	–	*<0.001*	
100–499 Y	2.2	(1.3–3.7)		9.3 (3.3)
<100 Y	2.8	(1.7–4.9)		14.6 (3.7)
Nomad occupation				
Yes	1.0	–		
No	2.1	(1.3–3.5)	0.004	22.7 (7.7)
Child's hygiene				
Uses soap	1.0	–		
Never/no soap	1.8	(1.2–3.0)	0.011	16.7 (6.5)
Presence of basic food				
3 items	1.0	–	*<0.001*	
2 items	1.6	(1.0–2.7)		9.6 (5.3)
0–1 item	3.3	(1.6–6.6)		6.8 (2.1)
No information	7.3	(2.6–2.1)		4.6 (1.2)
Flour origin				
From other village	1.0	–		
Only own production/none	2.7	(1.6–4.4)	<0.001	24.7 (6.2)
Presence of colza				
No	1.0	–		
Yes	1.9	(1.2–2.9)	0.008	7.8 (2.9)
Storage room type				
Other	1.0	–		
Tent	5.9	(2.0–1.8)	0.002	3.1 (0.8)
Water storage container				
Big	1.0	–	*<0.001*	
Medium	1.8	(1.1–2.9)		5.1 (2.3)
Small	3.5	(2.0–6.1)		8.8 (2.0)
Total (*n*=575)				98.9 (0.6)

[a] Adjusted odds ratio with 95% confidence interval
[b] *P*-values in italics are *P*-values for trend for ordinal variables
[c] AF: adjusted population attributable fraction with standard error

Water storage and analyses

KBD families more frequently had small water storage containers. In order to verify whether this association was more than an indicator of lower socio-economic status among KBD families, water samples were analysed for organic matter (TOC) levels. Mean log (TOC) was 0.47 in big containers, 0.49 in medium size containers and 0.95 in small containers ($P<0.001$). The frequency of cleaning the containers and the macroscopic appearance of the drinking water was not associated with KBD.

Grain storage

Grains were stored in a separate room by 65% of the families, in the living room by 36% and in other places (outside or in a tent) by 9%. The storage of grain in a tent was associated with KBD. Most storage room parameters (ventilation of the storage room, wall humidity, the type of storage containers used for grain and flour, storage of bags higher than the floor) were not associated with the disease, except for the type of floor in the storage room. Grain humidity was not related to the disease during May 1995 (mid-storage), but the humidity of recently stored grain (during October 1995) was higher in KBD families.

Mycological analyses were done on 60 barley grain samples obtained in October 1995 and detailed results are reported elsewhere [7, 9]. Three fungal taxa were independently associated with KBD (*Trichothecium roseum*, *Alternaria* sp. and *Dreschlera* sp.). *Cladosporium* sp. was also associated with KBD in multivariate analysis [2], but was considered only to be a background contaminate. A mycological index was produced for the three associated fungal taxa. The KBD prevalence rate increased dramatically from 13.1% if none of these taxa were isolated, to 51.3% if one and 88.5% if two or more taxa were isolated.

Table 3 Multivariate model for Kashin-Beck disease in Tibet, including mycological data

Variable	Adj. OR[a]	(95% CI)	*P*-value	AF% (SE)[b]
Gender				
Male	3.7	(1.0–14.5)	0.057	16.5 (8.2)
Age			<0.001	
8–11 years	11.2	(2.1–60.5)		17.1 (5.3)
12–15 years	66.2	(10.0–437.9)		38.1 (7.2)
Family income				
<500 Y	10.9	(2.4–50.2)	0.002	31.3 (8.6)
Presence of basic food				
0–1 item	19.1	(3.0–121.7)	0.002	10.8 (2.9)
Mycological index (ATD)[c]				
0 fungi	1.0	–	<0.001	
1 fungus	21.0	(4.0–109.6)		25.9 (5.6)
2–3 fungi	437.1	(45.4–4936)		38.9 (4.6)
Total (*n*=126)				99.9 (0.1)

[a] Adjusted odds ratio with 95% confidence interval
[b] AF: adjusted population attributable fraction with standard error
[c] Mycological index for presence of *Alternaria* sp., *Trichothecium roseum* and *Drechslera* sp.

Multivariate analysis

Model without mycological data

Eleven variables were independently associated with KBD (*n*=575; Table 2). Missing value categories were removed from the model except for the presence of basic food items in the household. Overall, the model correctly classified 73% of the subjects. The area under ROC curve was 0.80.

The adjusted population attributable fraction (AF) takes into account both the prevalence of the risk factor and the magnitude of the risk (OR) and therefore shows the impact of each factor on the disease. Included factors were, in descending order of impact: age, absence of any flour from other villages, family income, the absence of nomadic activities, male gender, child's hygiene, absence of either barley, wheat or flour, iodine deficiency <2.0 µg/l and the type of water container. Less important were the presence of colza and the storage of grain in a tent.

Model including mycological data

The mycological index was entered in the final model described above (*n*=126; Table 3). Seven non-significant variables were subsequently removed. Gender was kept for adjustment reasons. The AF for contamination of grain by *Trichothecium roseum*, *Alternaria* sp. or *Dreschlera* sp. was as high as 64.8%. Other included variables in descending order of impact were age (>7 years), family income (<500 Yuan per year), male gender and the presence of basic food items. The mycological model correctly classified 87% of the subjects. The area under ROC curve was 0.95, showing an excellent predictive value.

Adding the interaction between the mycological index and the urinary iodine resulted in an unstable model due to empty categories. There was a marked difference in the effect of iodine deficiency according to the contamination of the barley grains by *Alternaria* species. As shown in Fig. 1, the "protective" effect of iodine disappeared in the presence of *Alternaria*.

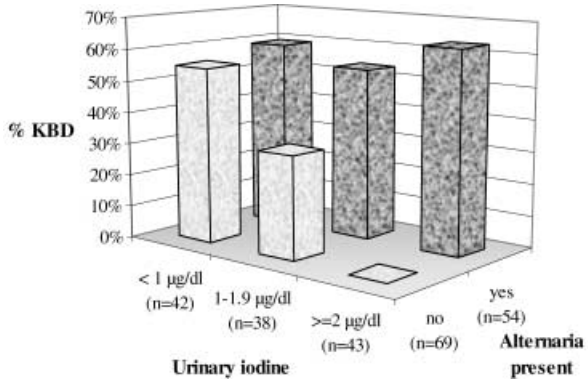

Fig. 1 Modification of the effect of urinary iodine on Kashin-Beck disease by grain contamination with *Alternaria* sp.

Discussion

We measured the individual and environmental characteristics likely to explain differences in the occurrence of Kashin-Beck disease between villages and also between people living in the same village. We assumed that most village-related differences were taken into account by the study design and therefore did not stratify results by village; consequently confounding by unmeasured village-related variables needs to be considered.

We used a clinical case definition as the number of mis-classified individuals was expected to be lower than occurred with the radiological patient definition [8]. Analysis of the radiological data showed a high proportion of normal radiographs among patients with KBD. Moreover, previous studies have shown good reproducibility of the clinical findings (kappa statistic >0.85, results not shown).

The difficulty of the clinical case definition, however, is the "grey zone" between the advanced cases and healthy children. As KBD is a slowly progressing disease,

Table 4 Causal model of Kashin-Beck disease in Tibet

Increased exposure to toxins	Effect on target cells (chondrocytes, bone cells)	Decreased defence mechanisms
Free-radical carriers: Mycotoxins from storage grains, Organic matter in drinking water	Oxidative damage → cell dysfunction, cell death	Decreased antioxidant defence: Selenium deficiency, Low dietary antioxidants (Vit C, Vit E)
Alternaria mycotoxin: competitive binding on thyroid hormone receptor	Decreased thyroid-hormone stimulated bone repair	Iodine deficiency → hypothyroidism
	?	Protein-calorie malnutrition

children at risk will develop more symptoms as they grow older with, as a consequence, a higher prevalence rate in the older age groups. But it can be very difficult to judge clinically when a joint has 'passed' from normal to 'deformed' or 'enlarged' and this may have caused mis-classification of some healthy individuals. Thus it is possible that more boys may have been classified as having KBD as they tend to have larger joints than girls. This could partly explain the apparent association of KBD with the male gender. Another possible explanation is that boys expose themselves more to a currently unknown risk factor that was not included in this study.

Individuals were at a greater risk of developing KBD if they had a brother or sister with the disease. This was to be expected as members were exposed to the same environmental risk factors. It is also possible that genetic factors could account for familial clustering, but these were not included in this study.

Most risk factors identified in our study can be interpreted according to the causal model of KBD presented in Table 4. It is possible that mycotoxins from fungi-contaminated stored grain and organic matter (fulvic acid) in drinking water act through a common mechanism by producing free radical-mediated damage to chondrocytes or bone cells under the condition of deficient antioxidant defences (oxidative stress), iodine deficiency and possibly protein-calorie malnutrition.

The marked differences in the degree of fungal contamination of stored barley between affected and unaffected families supports the hypothesis that mycotoxins are an important, if not the most important, determinant of KBD in Tibet (being responsible for 65% of our cases). The prevalence rate increased with the number of fungi isolated, and this suggests a cumulative effect of different taxa on the development of the disease. This observation also supports the hypothesis that different mycotoxins act through the same mechanism of free radical-mediated damage. This would also allow other fungal species to be involved at different moments in time or in other areas.

However, our data also suggest that binding of a mycotoxin to a specific receptor may constitute a complementary mechanism for mycotoxicity in KBD. The observation that KBD prevalence rates did not decrease with increasing urinary iodine levels in the presence of *Alternaria* sp. might be explained by the binding of a specific mycotoxin to a thyroid hormone receptor in bone cells [1].

With regard to the role of organic matter in drinking water, our study only showed that the fulvic acid hypothesis cannot be excluded in Tibet. The size of water containers was related to the disease, and this was independent of socio-economic status. We found that the organic matter content was higher in small water containers and this relationship between organic matter content and water container size (diameter) has been confirmed in a later study carried out in 1998. However, the nature of the molecules that are responsible remains to be identified.

Selenium levels did not vary between cases and healthy subjects in the study population. However, selenium was deficient in all our subjects, and thus could be an essential underlying factor. Selenium is involved in antioxidant defence mechanisms through the enzyme gluthathione peroxidase (GSHPx) which contains selenium at its active site and removes hydrogen peroxide. GSHPx levels were also very low in the study subjects [8]. Furthermore, the geographical distribution of Kashin-Beck disease coincides with the severe selenium deficient belt passing from north-east to south-west China.

Other antioxidants such as vitamins C and E have been shown to decrease the cytotoxicity of mycotoxins [5, 11, 12]. The observed associations of KBD with socio-economic status confirm that the protective role of other nutritional factors should be considered. The diet of the Tibetan rural population is very monotonous with access to fruits and vegetables being limited to a few months each year. Wealthier individuals can afford to buy other foods from merchants or in the cities, but the poorer families are almost entirely dependent on food that they produce themselves. A diversified diet acts through two mechanisms as a possible inhibitor of KBD: first, by the increased intake of dietary antioxidants, and secondly by decreased exposure to fungi-contaminated stored grain. Associations with family income, the presence of several basic food items in the household and the presence of flour originating from other villages are compatible with these mechanisms. Nomadic activities probably indicate specific differences in diet through higher consumption of meat and dairy products and a higher degree of barter with animal products. The less straightforward factor 'washing using soap' is probably just an additional indicator of a higher socio-economic

status. More children who washed themselves with soap also attended school where the diet was not entirely the same as at home.

A protective effect of food substances with high protein content with respect to KBD has been reported in an earlier publication [15]. In our study the association with thyroxine-binding globulin could be accounted for partly by protein-calorie malnutrition. Inadequate food and a low socio-economic status also result in chronic malnutrition. However, whether protein-calorie malnutrition is just a marker of the disease or is really important in the pathogenesis of KBD remains unclear.

Our study was the first to show an association of KBD with iodine deficiency [8]. Iodine-containing thyroid hormones stimulate both osteoblasts and osteoclasts and are important regulators of bone remodelling [6]. Hypothyroidism probably leads to impaired repair mechanisms in KBD and therefore adds to the clinical expression of the disease. In the presence of *Alternaria* mycotoxin(s) this repair mechanism may be blocked (Fig. 1).

A previous study that showed an effect of mycotoxins on iodine deficiency [13] could suggest that an association with KBD might in fact be a consequence of exposure to mycotoxins. However, iodine status parameters did not vary with the degree of fungal contamination of grains in our study.

Our study clearly indicates that KBD is a disease of poor farmer families. Although this disease will probably disappear with the rapid socio-economic development of China, specific preventive measures should be started in the remote rural areas where the prevalence of the disease is at its highest. However, the potential effect of preventive measures based on the multifactorial model should first be assessed in a preliminary trial. This should include measures to decrease the fungal contamination of grains at different stages of the agricultural cycle, and be combined with an increased protection based on two mechanisms: oxidative stress (antioxidant supplementation, e.g. selenium, vitamins E, C, A) and thyroid hormone-stimulated bone repair (iodine supplementation). This prevention trial should determine whether it is sufficient to correct only one factor in order to prevent the disease, or if several different preventive measures are necessary, with a cumulative protective effect.

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