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Ten years of follow-up of the Spanish cohort of the European PreventCD study: the lessons learned

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ABSTRACT

Aim: to evaluate the influence of gluten consumption on celiac disease development and to describe its natural history in the Spanish cohort of the European PreventCD study.

Methods: prospective multi-center double blind study of 225 children that were followed up from birth. All cases were HLA-DQ2/HLA-DQ8 positive with a 1st degree relative with celiac disease and were followed up in three centers from Madrid, Reus and Valencia. Gluten intake was determined between four and ten months according to the protocol. Gluten intake was ad libitum between eleven and 36 months and was prospectively quantified by means of dietary records. Clinical visits and specific antibody analysis for celiac disease were performed periodically.
Results: twenty-six cases were diagnosed, all had a positive biopsy and serology; 21 had gastrointestinal symptoms and five were asymptomatic. In addition, 2,565 food records were analyzed and statistically significant differences ($p < 0.001$) were found with regard to gluten consumption among the three centers, although not between celiac and non-celiac children ($p = 0.025$). The HLA-DQ2.5/DQ2.5 and DQ2.5/DQ2.2 genotypes had a relative risk of 4.7 (95% CI: 0.80-27.55; $p = 0.08$), which was higher than for the rest of genotypes. Female gender also had a relative risk that was five times higher than that for males.

Conclusions: the amount of gluten intake between eleven and 36 months or the duration of breast feeding were not risk factors for the development of CD in the Spanish population. The HLA genotype and gender were the most relevant associated factors. In this at-risk group, the disease presented before two years of age in the majority of the cases with a weak clinical expression.

Key words: Celiac disease. Gluten. Pediatric population.

INTRODUCTION

Celiac disease (CD) is an immunology-based systemic disorder that is triggered by the consumption of gluten in genetically susceptible subjects (HLA-DQ2 and/or HLA-DQ8 positive). It is characterized by the presence of specific antibodies, intestinal villi atrophy and a wide spectrum of clinical manifestations. These range from gastrointestinal symptoms of a diverse severity and/or extra-intestinal to asymptomatic cases, making early diagnosis difficult (1). Additionally, the low clinical expression form of the disease is usually associated with the adulthood stage and infrequently described in children (2,3). On the other hand, the HLA-DQ2/DQ8 genotype is widely distributed in the general population. However, only 1/3 of genetically susceptible individuals eventually develop the disease. Thus, the incidence is approximately 1% of the general population (4,5). The estimated prevalence is higher in first and second degree relatives of affected individuals, around 7%. This ranges from 2 to 7% in Spain. However, these studies have only been performed in adult populations. Therefore, there is no estimate of the prevalence in the at-risk pediatric population (6-11). Due to the differences in the prevalence of CD, several
classical observational studies suggested the need to assess determinant environmental factors with an essential role in the etiopathology of the disease, especially the age of gluten introduction into the diet and the duration of breast feeding (12-18). However, two recent prospective studies with a high level of scientific evidence have shown that the early or late introduction of gluten and the duration and type of breast feeding do not have a preventive effect on disease development. This goes against the previously posed hypothesis (19-22). Other retrospective studies have analyzed whether the intake of large amounts of gluten (both when introduced into the diet and during the two first years of life) could increase the risk of developing CD. A Swedish case control prospective study of a pediatric cohort followed-up until the age of two addressed this point. This study showed that cases diagnosed during the follow-up period consumed more gluten prior to seroconversion of the CD specific antibodies (23).

The European project PreventCD is a prospective study that has evaluated the impact of the early introduction of gluten into the diet. This is a randomized double blind study with the main objective of assessing the incidence of CD at three years of age after the early introduction of gluten. The European cohort is composed of pediatric subjects from eight countries (including Spain) at genetic risk of CD who were recruited and followed up prospectively from birth. The study was initiated in 2007 and the double blind arm was opened in 2013. The results of the follow-up until three years of age were published in 2014 (20). The follow-up of the Spanish cohort is still on-going; this cohort has been used to address the objectives of the present study. These included the analysis of the prevalence of CD and a description of the characteristics of cases in a pediatric at-risk cohort during a ten-year follow-up from birth. Furthermore, the gluten intake pattern during the first three years of life was assessed to determine whether it had a late effect on the development of CD.

MATERIAL AND METHODS

Subjects and study design

All the subjects included in the study were part of the Spanish cohort of the European PreventCD Project. Therefore, the inclusion and exclusion criteria, the frequency of the clinical visits, blood sample extraction, CD diagnosis, the estimation of the sample size
and the method used for patient randomization were the same as that established in the protocol of the main study (20,24).

According to the main protocol, the subjects were randomized at four months of age to receive either 100 mg of gluten daily (group A) or placebo (group B) up to six months of age. After the intervention, all parents were thoroughly instructed to progressively increase gluten intake as follows: 250 mg of gluten was recommended at six months, 500 mg at seven months, 1,000 mg at eight months and 1,500 mg of gluten at nine months. From ten months onwards, free gluten consumption was allowed and the mean daily gluten intake (MDGI) was quantified prospectively at 11, 12, 14, 16, 18, 20, 22, 24, 28, 30, 34 and 36 months of age. This was performed using food frequency questionnaires (FFQ) that were previously developed and validated in our population (23). The MDGI was calculated by multiplying the number of grams of vegetable proteins of each reported product by 0.8, according to a generally accepted method (25-28).

All children were periodically monitored every 2-3 months via a physical exploration, serum antigliadin, tissue transglutaminase antibodies (anti-TTG), total IgA in serum and symptoms of CD. The diagnosis of CD was confirmed according to the criteria of the European Society for Pediatric Gastroenterology, Hepatology and Nutrition 2012 (ESPGHAN) (29). A biopsy was taken when there were positive anti-TTG levels and/or symptoms of CD, according to the protocol of the main study (20,24).

The study was approved by the local medical ethical committee of the participating centers.

**Statistical analysis**

Data were summarized using the mean and standard deviation (SD). The mean daily gluten intake values among the centers, as well as the means of CD and non-CD cases and male and female subjects, were compared using the t-test. A multivariable Cox proportional hazards regression was performed for each individual predictor in order to assess the hazard risk of each independent variable and the probability of developing CD. The variables included in the statistical model were city, gender, increase of gluten consumption per unit (gram), HLA risk group and intervention group (gluten or placebo). The p values for the mixed models were estimated using the
Satterthwaite test with the appropriate degrees of freedom, and differences were considered as significant when p < 0.05. All statistical analyses were performed using the R software (version 3.1.2).

RESULTS
The Spanish cohort of the PreventCD study included 225 children recruited from hospitals in Valencia, Madrid and Reus. At the time of the analysis, all the children were older than six years of age, 60% were older than eight years and 26 children had been diagnosed with CD (11.5%) (Table 1 and Fig. 1).

Environmental factors: gluten intake and breastfeeding
A total of 2,565 CFC were analyzed (up to 12 food registers per child); these results are presented in figure 2. From eleven months onwards, when the gluten consumption was unrestricted, significant differences in the MDGI were observed among the cities at different ages (p < 0.001). A lower consumption was found in Reus and a higher consumption in Valencia. However, the MDGI at different ages was similar among the children who did and did not develop CD (p = 0.25) (Table 2). The MDGI among boys and girls was also similar and no statistically significant differences were found in the general analysis (p = 0.29). There were no differences between boys and girls from the same center (p = 0.18). Finally, no statistically significant differences were found among the centers with regard to the duration of breastfeeding (p > 0.05). The mean duration was eight, seven and six months in Madrid, Reus and Valencia, respectively. Moreover, the duration of breastfeeding was also similar among the children who developed CD (mean of six months) and those who did not develop CD (mean of five months).

CD development risk
In addition to the descriptive analysis, the risk of CD development was analyzed using the Cox proportional hazard model, adjusting for the different variables. The results obtained with the analysis of HLA and gender are summarized in table 3. Out of five established risk groups, the HLA group 1 (DQ2.5/DQ2.5 and DQ2.5/DQ2.2) showed a four-fold higher risk. The hazard ratio was 4.7 (95% CI: 0.80-27.55) and the p-value was
close to the statistical significance (p = 0.08). As shown in table 1, 13 of 26 CD cases belonged to group 1, i.e., 50% of the total cases. Male gender had a five-fold lower risk than female gender. The hazard ratio was 0.18 (95% CI: 0.03-1.16) and the p-value was close to the statistical significance (p = 0.07). The progressive increase of gluten consumption had a hazard ratio of 1.11 (95% CI: 0.69-1.78) but was not statistically significant (p = 0.66). There was also no significant association with CD development and the other variables studied (city and intervention group).

**Characteristic of CD cases**

All the 26 CD cases were diagnosed via an intestinal biopsy. All cases had Marsh 3 grade according to the Marsh Oberhuber classification and positive anti-TTG values prior to the biopsy. The median age of positive serology onset was 24 months (range, 12-94) and there were no cases of IgA deficit.

With regard to clinical forms of expression, 21 cases had gastrointestinal symptoms, of which 16 had diarrhea with at least one or more associated symptoms (abdominal pain/distension and/or vomits). The remaining five symptomatic cases had constipation and abdominal pain and there were some cases of anorexia. Severe nutrition was not observed in any of the 21 cases. Of the five asymptomatic cases, only one case had iron deficiency without anemia.

With regard to the age at diagnosis, ten children were diagnosed before 24 months of age (two asymptomatic cases) and nine cases, between 25 and 36 months of age. Therefore, 73% of cases presented the disease before three years of age. The remaining seven cases were diagnosed after three years of age (three asymptomatic cases). The median age at diagnosis (time of biopsy) was 26 months (range 14-96).

The most relevant data were found in the distribution by gender and HLA risk group. Thirteen cases of the 26 diagnosed cases belonged to the group 1 (homozygous) of which eleven cases were diagnosed before three years of age and two cases, between three and four years of age. In the HLA risk group 3, seven of eleven cases were diagnosed before three years of age and four cases, between four and eight years of age. Finally, the remaining two cases belonged to HLA risk group 2; one case was diagnosed at 27 months of age and the other, at four years of age. No cases were diagnosed from the HLA risk groups 4 and 5. With regard to the distribution of the
Spanish cohort by HLA risk group (Table 1), 25% of the 51 homozygous children developed CD, whereas only 11% of the 96 children from the HLA risk group 3 developed CD and only 10% of children in the HLA risk group 2 developed CD.

Finally, in relation to gender, 18 of 26 diagnosed cases were girls (70%) and all were diagnosed before four years of age, except one case which was diagnosed at eight years of age. On the other hand, 10/18 cases belonged to the HLA risk group 1, 1/18 to group 2 and 7/18 cases were from HLA risk group 3. Furthermore, 14 of 18 received gluten during the intervention period and four received the placebo between four and six months of age. Of the eight males diagnosed, three were from HLA risk group 1 and only one case was from group 2, and four cases were from HLA risk group 3. Furthermore, 5/8 cases were diagnosed between 20 and 30 months of age and the remaining at four, five and seven years of age. Finally, 6/8 cases received the placebo during the intervention period and two children received gluten.

The most remarkable result is that, from the entire cohort of 225 children, 51 were homozygous (24 girls and 27 boys). Only three of the 27 boys developed CD (11%), whereas ten of the 24 girls developed CD (40%) and were diagnosed before four years of age. Therefore, the combination of HLA DQ2.5/DQ2.5 and DQ2.5/DQ2.2 and female gender is a risk factor for early onset of celiac disease.

**DISCUSSION**

Our results show that the consumption of gluten during the first three years of life is not a risk factor for CD development in the pediatric population at genetic risk, at least up to the age of six years. In addition, this is the first study to show the close relationship between HLA, gender and CD risk in a pediatric population.

The cohort with a genetic risk was followed up from birth to CD diagnosis. We have shown that the disease can present very early, especially in DQ2 homozygotic subjects (HLA-DQ2.5/DQ2.5 and DQ2.5/DQ2.2), as 13 cases were diagnosed before the age of four. Moreover, this genetic group has a four times greater risk for CD and represents 50% of the diagnosed cases, but only 22% of our cohort. These results are similar to other European and American cohorts, which have shown that this genotype is one of the key factors for the development of the disease. Furthermore, the greater risk of CD development in homozygotic subjects was expressed at very early ages (20,21,30,31).
The global prevalence of CD cases in our cohort is higher than in other Spanish cohorts, i.e., 11.6% vs an estimated 7% (7-11). None of the previous series included a pediatric population, nor did they specify the distribution of the HLA risk groups. Our study suggests that we have already diagnosed the majority of CD cases in our cohort, even though the cohort was followed-up to the age of 6-8 years. The number of newly diagnosed cases in other studies may be an oligo or asymptomatic form of presentation in the pediatric age and were not registered. Therefore, the global prevalence in these series is underestimated. Likewise, this difference could be due to a greater proportion of homozygous HLA subjects in our cohort as compared to the other Spanish cohorts. In fact, the number of homozygous HLA cases varies significantly between the three participating centers in the study.

Our results also confirm that females have a five-fold higher risk for the development of CD as compared to boys. This is in accordance with other studies that have shown that girls have a higher risk from a younger age (20,32-34). However, the factors responsible have not been established. Hormonal factors that are associated with the differences in the prevalence according to gender in other diseases are not applicable in early childhood. In addition, it is important to note that from the total cohort, 40% of homozygous females were diagnosed with CD during the follow-up period. All before the age of four years, except for one case. This means that the combination of the homozygous genotype with female gender is a risk factor of a very early presentation of the disease. Therefore, it must be taken into account for the follow-up of the at-risk pediatric population.

With regard to the clinical forms of expression, although the majority of subjects presented characteristic gastrointestinal symptoms in our cohort, five were asymptomatic at the time of diagnosis. Therefore, the asymptomatic form of CD is not exclusive to older child or adolescents, as it has been classically considered (3). On the other hand, all children diagnosed with CD tested positive for antibodies against transglutaminase. This includes the six subjects diagnosed before the age of two; seroconversion was demonstrated between 12 and 18 months of age in these cases. Previous studies report a rate of negative anti-TTG in children younger than 18 months of 17% (35) and recommend the use of antigliadin antibodies for CD screening in
children younger than 2-3 years. However, the number of cases in our cohort is low. On the other hand, our data is consistent with that reported in other European cohorts, in which all the cases tested positive for anti-TTG at diagnosis, independently of age (20,21).

With regard to the importance of the amount of gluten consumed during the development of CD, the confidence interval of the variable “gluten intake” was 0.69-1.78. The progressive increase of gluten intake between eleven and 36 months could reduce or increase the risk of CD development. However, this was not significant in any case. Moreover, the registered intake in the CD and non-CD cases was similar. The importance of gluten, both at the time of introduction into the diet and the amount consumed, as well as its relationship with CD risk have been widely debated during recent years. The recommendations from the Nutrition Committee of ESPGHAN from 2008 are based on observational studies (2,4-6). These guidelines suggested that introducing gluten in small amounts, preferably during the breast feeding period, could reduce the risk of CD. However, the latest studies have proven that the age of introduction does not significantly influence the development of the disease in at risk populations. In 2016, an expert committee of ESPGHAN updated these recommendations, indicating that gluten can be introduced at between four and 12 months of age (22). In contrast, there is no consensus with regard to the optimal amount of gluten introduced into the diet. This is due to the fact that there are few prospective studies that address this point, and thus establishing specific recommendations is not possible at this time.

The retrospective studies referred to in this paper have formed the basis of the 2008 recommendations. These studies were based on the Swedish epidemic which suggested that the ingestion of larger amounts of gluten at the time of gluten introduction into the diet increased the incidence CD before the age of two (4-6). According to the Swedish epidemic data, the rise in CD cases during the time of the epidemic between 1987 and 1995 was attributed to different factors. These included the fact that daily gluten consumption in children younger than two years of age during the epidemic was twice the limit (4.5 grams per day) compared to the pre-epidemic period (2.5 grams per day). If we compare these amounts with our results, the daily gluten consumption at two years of age was 5, 4.39 and 3.9 grams in
Valencia, Madrid and Reus, respectively, compared with the 4.5 grams consumed during the Swedish epidemic period. If a high consumption of gluten during the first years of life increases the risk of developing CD before two years of age, in theory Valencia should have more cases of CD. However, this was not observed even though the Valencia cohort had the highest gluten consumption. Furthermore, the Reus cohort had the lowest gluten consumption at any age and also the highest percentage of CD cases, as well as the greatest number of HLA-DQ2 homozygous children.

The nested Swedish case-control study, TEDDY, is the only prospective study with a similar cohort to ours. The findings in this study also differ from our data (23). The authors concluded that the Swedish CD cases consumed a larger amount of gluten (4.9 grams) prior to seroconversion to positive antibodies as compared to the control population (3.9 grams). However, the TEDDY study categorized gluten intake by tertiles and used arbitrarily cut-off points as follows: low (< 3.4 g/d), medium (3.4-5.0 g/d) and high (> 5.0 g/d). Therefore, this study assumed that those children who did not consume gluten (0 grams) would have the same risk as those children that consumed 3.4 grams. The categorization of continuous variables is an important limitation that leads to the loss of information as well as other well established bias and shortcomings (36,37). This method of gluten intake estimation could explain the discrepancies with our results.

The main limitation of our study is the fact that subjects received a specific intervention between four and six months of age and a staggered introduction of gluten up to ten months of age. This is not representative of gluten introduction into the diet in clinical practice. However, the age of gluten introduction, either early or late, and the amount of gluten consumed during the first three years of life are not risk factors for CD development. Therefore, we can consider that the intervention performed in these subjects did not significantly interfere with the natural history of CD. On the other hand, the strength of our study is the large pediatric cohort, the follow-up using a common standardized protocol, the high number of FFQ analyzed and the geographical distribution of the sample. All these factors allow our data to be extrapolated to the general population.

In conclusion, an early screening program in patients with at least one first-degree relative diagnosed with CD is justified. This is due to the fact that the HLA genotype
and gender are demonstrated determinant factors in CD development. The identification of individuals at high risk would allow regular monitoring, mainly of DQ2 homozygous children, and therefore facilitate diagnosis of the disease. In addition, these results could be useful to identify a target population to apply new tools for early diagnosis (38, 39). Currently, there are no specific recommendations from official institutions with regard to the follow-up of risk population. However, according to our results and previous studies, annual clinical and serological controls seem appropriate. Therefore, this could avoid the clinical consequences of a late diagnosis, avoid complementary tests and unnecessary health expenses. Furthermore, this could prevent the suffering of both the pediatric patient and their family.

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REFERENCES


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### Table 1. Characteristics of the study population

<table>
<thead>
<tr>
<th>City</th>
<th>n*</th>
<th>Intervention group†</th>
<th>Gender</th>
<th>HLA risk group‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Gluten/Placebo</td>
<td>ө/ө†</td>
<td>Group 1</td>
</tr>
<tr>
<td>Madrid</td>
<td>63</td>
<td>34/29</td>
<td>39/24</td>
<td>9</td>
</tr>
<tr>
<td>Valencia</td>
<td>75</td>
<td>37/38</td>
<td>45/30</td>
<td>16</td>
</tr>
<tr>
<td>Reus</td>
<td>87</td>
<td>45/42</td>
<td>43/44</td>
<td>26</td>
</tr>
<tr>
<td>Total cohort</td>
<td>225</td>
<td>116/109</td>
<td>127/98</td>
<td>51 (22.6%)§</td>
</tr>
<tr>
<td>CD cases</td>
<td>26</td>
<td>16/10</td>
<td>18/8</td>
<td>13 (25%)‖</td>
</tr>
</tbody>
</table>

CD: celiac disease. *Total number of children included in each center and diagnosed with CD. †Number of children that received gluten or placebo between week 16 and 24 from the total cohort and CD cases. HLA genotype, group 1 included DQ2.5/DQ2.5 and DQ2.5/DQ2.2; group 2, DQ2.2/DQ7; group 3, DQ2.5/DQ7, DQ2.5/DQ8 and DQ2.5/X; group 4, DQ2.2/DQ2.2, DQ2.2/DQ8 and DQ8/DQ8; and group 5, DQ2.2/X, DQ8/DQ7 and DQ8/X. §Percentage with respect to the total cohort of 225 children. ‖Percentage with respect to the subjects from each HLA group.
Table 2. Mean daily gluten intake calculated from the food records in CD and non-CD cases

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>Global</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>CD (n = 24)</td>
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<tr>
<td></td>
<td>MDGI (SD)</td>
</tr>
<tr>
<td>11</td>
<td>2.59 (1.56)</td>
</tr>
<tr>
<td>12</td>
<td>3.65 (1.63)</td>
</tr>
<tr>
<td>14</td>
<td>3.73 (1.33)</td>
</tr>
<tr>
<td>16</td>
<td>4.06 (1.98)</td>
</tr>
<tr>
<td>18</td>
<td>4.52 (2.03)</td>
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<tr>
<td>20</td>
<td>4.54 (1.72)</td>
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<td>22</td>
<td>4.70 (1.82)</td>
</tr>
<tr>
<td>24</td>
<td>5.23 (2.85)</td>
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<tr>
<td>28</td>
<td>4.78 (2.03)</td>
</tr>
<tr>
<td>30</td>
<td>5.60 (2.35)</td>
</tr>
<tr>
<td>34</td>
<td>5.96 (2.22)</td>
</tr>
<tr>
<td>36</td>
<td>5.82 (3.38)</td>
</tr>
</tbody>
</table>

MDGI: mean daily gluten intake in grams per day; SD: standard deviation; CD: cases diagnosed with celiac disease; Non-CD: healthy subjects.
Table 3. Effect of the variables included in the multivariable regression model on the development of celiac disease. Hazard ratios (95% CI) and p-values

<table>
<thead>
<tr>
<th>Variable</th>
<th>HR (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>City</td>
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<td></td>
</tr>
<tr>
<td>Madrid</td>
<td>1.0 (reference)</td>
<td>-</td>
</tr>
<tr>
<td>Reus</td>
<td>0.9 (0.17-4.55)</td>
<td>0.90</td>
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<tr>
<td>Valencia</td>
<td>0.1 (0.04-3.01)</td>
<td>0.20</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>1.0 (reference)</td>
<td>-</td>
</tr>
<tr>
<td>Male</td>
<td>0.2 (0.02-1.16)</td>
<td>0.07</td>
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<tr>
<td>Intervention group</td>
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<td></td>
</tr>
<tr>
<td>Gluten</td>
<td>1.0 (reference)</td>
<td>-</td>
</tr>
<tr>
<td>Placebo</td>
<td>0.9 (0.72-1.82)</td>
<td>0.66</td>
</tr>
<tr>
<td>HLA risk group</td>
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<td></td>
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<tr>
<td>Group 1</td>
<td>4.7 (0.80-27.5)</td>
<td>0.08</td>
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<tr>
<td>Group 2</td>
<td>3.2 (0.21-22.0)</td>
<td>0.32</td>
</tr>
<tr>
<td>Group 3</td>
<td>1.0 (reference)</td>
<td>-</td>
</tr>
<tr>
<td>Group 4</td>
<td>1.7 (0.05-53.4)</td>
<td>0.75</td>
</tr>
<tr>
<td>Group 5</td>
<td>0.4 (0.01-14.0)</td>
<td>0.64</td>
</tr>
<tr>
<td>Gluten intake</td>
<td>1.1 (0.69-1.78)</td>
<td>0.66</td>
</tr>
</tbody>
</table>
Fig. 1. Flow chart of subject selection.
Fig. 2. Mean daily gluten intake (g/day) of the cohort from each city.