

RESEARCH LETTER

Serum zonulin is associated with presence and severity of atopic dermatitis in children, independent of total IgE and eosinophil

Y. H. Sheen¹ | H. M. Jee² | D. H. Kim³ | E. K. Ha² | I. J. Jeong³ | S. J. Lee² |
H. S. Baek⁴ | S. W. Lee⁵ | K.-J. Lee⁶ | K. S. Lee²  | Y.-H. Jung² | M. Sung⁷ |
M. A. Kim⁸ | M. Y. Han² 

¹Department of Pediatrics, CHA Gangnam Medical Center, CHA University, Seoul, Korea

²Department of Pediatrics, CHA Bundang Medical Center, CHA University, Seongnam, Korea

³Department of Dermatology, CHA Bundang Medical Center, CHA University, Seongnam, Korea

⁴Department of Pediatrics, Hallym University Kangdong Sacred Heart Hospital, Hallym University College of Medicine, Seoul, Korea

⁵CHA University, Seongnam, Korea

⁶Department of Information and Statistics, Korea National Open University, Seoul, Korea

⁷Department of Pediatrics, Inje University Haeundae Paik Hospital, Busan, Korea

⁸Department of Internal Medicine, CHA Bundang Medical Center, CHA University, Seongnam, Korea

Correspondence

Man Yong Han, Department of Pediatrics, CHA University School of Medicine, Bundang-gu, Seongnam, Gyeonggi-do, Korea.

Email: drmesh@gmail.com

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To the Editor:

Atopic dermatitis (AD) is characterized by dry eczematous skin lesions with intense pruritus due to a hyperactive immune response and dysfunction of the skin barrier,¹ which consists of epithelial cells and tight junctions (TJs). TJs contribute to the barrier function of epithelial and endothelial cells and are therefore important for regulation of many physiological and pathological events.²

Zonulin is a 47 kDa protein that increases permeability of the small intestine, has a role in the intestinal innate immune system,³ and has higher expression in autoimmune diseases in which the dysfunction of TJ proteins plays an essential role.⁴ It is the only physiological protein known to reversibly modulate intestinal permeability via intercellular TJs.⁵ Furthermore, there is considerable evidence that TJs play a role in the pathophysiology of AD.⁶ However, no previous studies have examined the role of circulating zonulin in the development and severity of AD, the association of zonulin with clinical and laboratory factors in AD patients, or the secretion of zonulin by skin epithelial cells. Therefore, in this study, we compared serum zonulin concentrations of AD children and healthy controls, determined the association of clinical factors with the severity of

AD, and measured the production of zonulin by skin epithelial cells in AD subjects and healthy controls.

This cross-sectional study examined children aged 5-15 years from 3 kindergartens and 6 elementary schools in Seongnam, South Korea, in June through July of 2015 (Seongnam Atopy Project). A modified Korean version of the International Study of Asthma and Allergies in Childhood questionnaire⁷ was distributed to 5196 children. Subjects were excluded if the questionnaire was not returned ($n = 762$), or had incorrect answers or had chronic diseases ($n = 519$), or there were no written informed consent forms for the SCORing Atopic Dermatitis (SCORAD) or blood and/or skin tests ($n = 3351$). Among the remaining 564 children (14.4%), 51 (9.0%) refused blood sampling and 11 (2.0%) refused skin testing. An additional 247 children (43.8%) were excluded from healthy controls because they had SCORAD indexes >0 and/or had allergic diseases (allergic rhinitis or asthma). A total of 255 children (AD: 115; and healthy controls: 140) were included in the final analysis. This study was approved by the institutional review board of the CHA Bundang Medical Center, CHA University (IRB No. 2015-05-075). Written informed consent was obtained from all parents or guardians.

The AD severity was classified according to the SCORAD index: mild (<25), moderate (25-50), and severe (>50).⁸ We collected blood

Sheen and Jee equally contributed to this study.

samples to measure white blood cell count, 25-hydroxyvitamin D, total eosinophil count (TEC), and total IgE level (Phadia AB, Uppsala, Sweden) and conducted skin tests to assess allergic sensitization to 22 allergens (Lofarma, Milan, Italy).⁹ Subjects were considered atopic if there was one or more positive reactions (wheal diameter >3 mm) to any allergen in the skin test. The zonulin concentration was measured in duplicate using a competitive enzyme-linked immunosorbent assay (ELISA) kit (K5600, Immundiagnostik AG, Bensheim, Germany). The threshold level was 0.45 ng/mL. The intra- and interassay coefficients of variation were <4% for these tests.

To evaluate the zonulin expression in the epidermis, 3-mm punch biopsies from the truncal area of three adults with AD and three healthy controls were subjected to immunohistochemical staining. The degree of stained zonulin in the tissue was quantified using a software (iSolution X64[®], iMTechnology[®], USA). All statistical analyses were performed using IBM SPSS Statistics ver. 22.0 (IBM Co., Armonk, NY, USA). All statistical tests were two-sided, and a *P* value below .05 indicated statistical significance.

The AD group had a greater median serum zonulin level than the control group (11.0 ng/mL [IQR: 8.2-16.1] vs 7.8 [IQR: 4.9-10.9]; *P* < .001). The AD group had a higher sensitization rate in the skin test with regard to *Dermatophagoides pteronyssinus*, *Dermatophagoides farina*, birch, and oak (all *P* < .05, Table S1). Analysis of all children indicated that serum zonulin level had significantly positive correlations with age ($\rho = 0.17$, *P* = .007), height ($\rho = 0.18$, *P* = .004), and the SCORAD index ($\rho = 0.28$, *P* < .001), but not with total IgE, TEC, or the number of allergens to which a child is sensitized. After adjustment for sex, age, BMI z-score, comorbid allergic diseases, parental history of allergic diseases, atopic sensitization, and 25-hydroxyvitamin D, each 1 ng/mL increase in serum zonulin was associated with a 9% greater risk of AD (adjusted odds ratio [aOR]: 1.09; 95% confidence interval [95% CI]: 1.02-1.16; *P* = .012). A subgroup analysis of the 115 AD subjects (86 children with mild AD, 26 with moderate AD, and 3 with severe AD) demonstrated that serum zonulin was significantly associated with the SCORAD index

($\rho = 0.27$, *P* = .004) and its intensity and subjective signs, but not with total IgE or TEC. The SCORAD index was significantly correlated with total IgE and TEC in the whole subjects and the AD subgroup analyses.

After adjustment for confounders and total IgE, each 1 ng/mL increase in serum zonulin was associated with a 15% greater risk of moderate-severe AD (aOR: 1.15; 95% CI: 1.03-1.27; *P* = .012; Model 1, Table 1). We obtained the same increased risk of moderate-severe AD after adjustment for the same confounders and TEC (aOR: 1.15; 95% CI: 1.04-1.27; *P* = .008; Model 2, Table 1). Children with zonulin in the third tertile were more likely to have moderate-severe AD than those with zonulin in the first tertile after adjustment for total IgE (*P* for trend = .028; Model 1) or TEC (*P* for trend = .016; Model 2).

The skin of healthy subjects (Figure 1A) and the non-lesional skin from AD subjects (Figure 1B) had little or no expression of zonulin. However, the epidermis of AD lesions had distinct staining of zonulin in the suprabasal and spinous layers (Figure 1C,D). The intensity and quantification of the zonulin staining were greater in subjects with moderate AD (Figure 1D) than those with mild AD (Figure 1C). This was also confirmed by the quantitative analysis of zonulin staining (Table S2).

This is the first study to report the association of serum zonulin level with the presence and severity of AD and the up-regulated zonulin expression in the impaired skin epithelial cells of the AD patients.

Defects in the skin barrier such as in the TJs induce penetration or reactivity to microbes, allergens/antigens, and irritants into the dermis and possibly promote Th2 immune responses, which is characteristic of early AD lesions. This suggests that structural defects in the TJs of AD patients may induce Th2 responses by enhancing the trafficking of non-self antigens.⁶ However, we found that the serum zonulin level did not correlate with total IgE, TEC, or atopic sensitization. Instead, we demonstrated an association between increased production of zonulin by skin epithelial cells of AD patients and skin

TABLE 1 Association between serum zonulin level and the severity of atopic dermatitis (N = 115)

	Unadjusted		Adjusted Model 1 ^a		Adjusted Model 2 ^b	
	OR (95% CI)	<i>P</i> value	aOR (95% CI)	<i>P</i> value	aOR (95% CI)	<i>P</i> value
Zonulin (ng/mL)	1.14 (1.07-1.24)	<.001	1.15 (1.03-1.27)	.012	1.15 (1.04-1.27)	.008
Zonulin tertiles						
Tertile 1 (4.5-9.3 ng/mL)	Reference		Reference		Reference	
Tertile 2 (9.4-14.7 ng/mL)	0.46 (0.13-1.67)	.24	0.47 (0.09-2.49)	.38	0.33 (0.07-1.67)	.18
Tertile 3 (14.8-29.6 ng/mL)	3.14 (1.15-8.59)	.03	3.76 (0.94-15.14)	.06	3.09 (0.87-11.01)	.08
<i>P</i> value for trend		.004		.028		.016

aOR, adjusted odds ratio; OR, odds ratio.

^aModel 1 adjusted for sex, age, body mass index z-score, comorbid allergic diseases (current asthma and current allergic rhinitis), parental history of allergic diseases (asthma, allergic rhinitis, and atopic dermatitis), atopic sensitization, 25-hydroxyvitamin D (deficiency, <20 ng/mL; insufficiency, 20-29.9 ng/mL; and sufficiency, ≥30 ng/mL), and total IgE.

^bModel 2 adjusted for sex, age, body mass index z-score, comorbid allergic diseases (current asthma and current allergic rhinitis), parental history of allergic diseases (asthma, allergic rhinitis, and atopic dermatitis), atopic sensitization, 25-hydroxyvitamin D (deficiency, <20 ng/mL; insufficiency, 20-29.9 ng/mL; and sufficiency, ≥30 ng/mL), and total eosinophil count. Significant *P* values (< 0.05) are in bold.

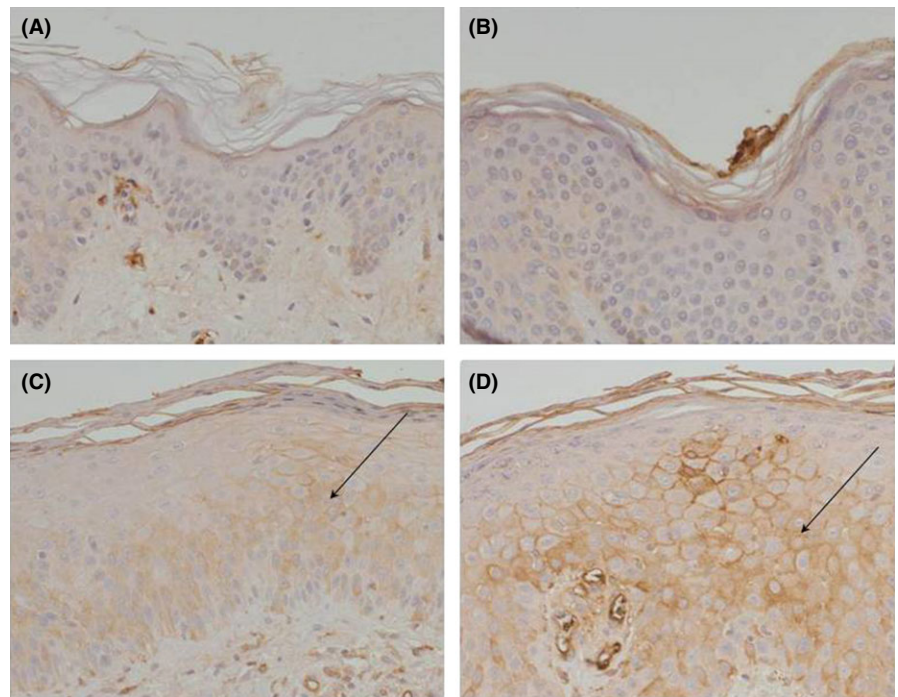


FIGURE 1 Representative immunohistochemical staining images ($\times 200$) showing the expression of zonulin (arrow) in the normal skin of a healthy adult control (A), non-lesional skin from an adult with AD (B), lesional skin from an adult with mild AD (C), and lesional skin from an adult with moderate AD (D)

damage or irritation. Given the very short half-life of zonulin⁵ and our finding that damaged skin cells secrete zonulin, we speculate that zonulin functions temporarily and locally and therefore has no association with total IgE, TEC, or atopic sensitization.

If chronic inflammation develops in response to environmental stimuli, it leads to up-regulated expression of zonulin,^{3,10} which then activates epidermal growth factor receptor (EGFR) through direct binding and/or transactivation of protease-activated receptor 2 (PAR2), ultimately leading to TJ disassembly¹⁰ of skin epithelial cells. However, we speculate that serum zonulin is not produced as the result of the systemic immune response for three reasons. First, the lesional skin specimens from the AD subjects produced zonulin, whereas the non-lesional skin specimens from AD subjects and normal skin specimens from controls produced minimal or no zonulin. Second, serum zonulin was significantly associated with AD severity after adjustment for allergic diseases. Third, serum zonulin was not associated with systemic Th2 biomarkers, such as total IgE and TEC.

The small intestines of mammalian model organisms exposed to enteric bacteria secreted zonulin only on the luminal side of the mucosa, followed by a decrease in small-intestinal tissue resistance, indicating that the zonulin-induced opening of the paracellular pathway of the gut epithelial cells may be a defensive mechanism in response to local inflammation caused by enteric bacteria.³ It is also plausible that TJ dysregulation, caused by up-regulated zonulin in the skin epithelial cells of genetically susceptible subjects, may lead to increased penetration or reactivity to microbes, allergens/antigens, and irritants, which then further impairs the epidermal barrier of the skin. Thus, we speculate that our finding of increased serum zonulin levels in AD subjects and upregulation of zonulin in the impaired skin epithelial cells from AD subjects may be a defensive mechanism where zonulin production may contribute to the host innate immune

response against bacterial colonization of the skin. However, in genetically susceptible subjects, such as those with AD, this response may be exaggerated and thereby impair skin barrier function and/or aggravate the disease.

This study has some limitations: a cross-sectional design, no measurement of TJ structural proteins, the comorbid allergic diseases of the subjects, the possibility that some of the study subjects may have had comorbid autoimmune or other chronic diseases, and no data on microbiota in the AD and healthy subjects.

We demonstrated that the serum zonulin is associated with presence and severity of AD. Further understanding of the causal relationships underlying these associations may lead to new therapeutic modalities targeting TJs and zonulin.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Jee HM, Lee KS, Jung YH, and Han MY designed the research. Jee HM, Kim DH, Jeong IJ, Lee SJ, Lee KS, Jung YH, and Han MY performed the research. Jee HM, Ha EK, Lee SJ, Lee KS, Jung YH, and Han MY collected the data. Sheen YH, Jee HM, Kim DH, Ha EK,

Jeong IJ, Lee SJ, Baek HS, Lee SW, Lee KJ, Lee KS, Jung YH, Sung MS, Kim MA, and Han MY analysed and interpreted the data. Sheen YH, Jee HM, Ha EK, Lee SJ, Baek HS, Lee SW, Lee KJ, Lee KS, Jung YH, Sung MS, Kim MA, and Han MY performed the statistical analysis. Sheen YH, Jee HM, Kim DH, Ha EK, Jeong IJ, Lee SJ, Baek HS, Lee SW, Lee KJ, Lee KS, Jung YH, Sung MS, Kim MA, and Han MY wrote/edited the manuscript. All authors read and gave the final approval of the version to be published.

ORCID

K. S. Lee  <http://orcid.org/0000-0002-6300-1348>

M. Y. Han  <http://orcid.org/0000-0002-9077-5779>

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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SUPPLEMENTARY TABLE 1. Comparison of allergens that the study subjects were sensitized to in the skin prick testing.

Allergens	Atopic dermatitis (n=115)	Healthy controls (n = 140)	P value
<i>Dermatophagoides</i>	54/113 (47.8)	41/140 (29.3)	.003
<i>pteronyssinus</i>			
<i>Dermatophagoides</i>	45/113 (39.8)	38/140 (27.1)	.03
<i>farina</i>			
Birch	19/113 (16.8)	11/140 (7.9)	.03
Oak	22/113 (19.5)	14/140 (10.0)	.03
Elm	5/113 (4.4)	4/140 (2.9)	.50
Hop	0/113 (0)	2/140 (1.4)	.20
Egg	3/113 (2.7)	0/140 (0)	.052
Milk	0/113 (0)	0/140 (0)	
Peanut	2/113 (1.8)	3/140 (2.1)	.83
Wheat	0/113 (0)	1/140 (0.7)	.37
Walnut	2/113 (1.8)	0/140 (0)	.11
Cod	1/113 (0.9)	0/140 (0)	.27
Pork	1/113 (0.9)	2/140 (1.4)	.69
Mussel	0/113 (0)	1/140 (0.7)	.37
Shrimp	2/113 (1.8)	2/140 (1.4)	.83
Apple	0/113 (0)	0/140 (0)	
Peach	0/113 (0)	0/140 (0)	
Kiwi	1/113 (0.9)	2/140 (1.4)	.69

Orange	1/113 (0.9)	2/140 (1.4)	.69
Tomato	0/113 (0)	1/140 (0.7)	.37
Strawberry	1/113 (0.9)	1/140 (0.7)	.88
Celery	10/113 (8.9)	7/140 (5.0)	.22

Subjects were considered sensitized if the wheal diameter was >3 mm to the respective allergen in the skin prick test.

Significant *P* values (<.05) are in bold.

SUPPLEMENTARY TABLE2. Quantitative analysis of the immunohistochemical staining of zonulin

Variables	Subject # 1	Subject # 2	Subject # 3
Normal skin from healthy controls	N-C	N-C	N-C
Non-lesional skin from AD subjects	N-C	N-C	N-C
Lesional skin from mild AD subjects	8.6%	6.4%	5.7%
AD lesional skin from moderate AD subjects	16.8%	15.4%	14.7%

The number in the table indicates the percentage of zonulin-expressing area in the 3-mm punch biopsied tissue obtained from the truncal area of each subject using a software (iSolution X64[®], iMTechnology[©], USA).N-C, Not checkable; AD, atopic dermatitis.