**Zonulin and blood-brain barrier permeability are dissociated in humans**

*Letter-to-Editor*

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# **Dear Editor,**

Zonulin, or prehaptoglobin-2, mediates intestinal permeability in coeliac disease through regulation of epithelial tight junctions.1 Tight junction breakdown at the blood brain barrier (BBB) is a common pathological finding in neurological disease2 and several *in vitro* and preclinical *in vivo* studies have suggested that zonulin plays a role in modulation of BBB permeability,3-6 yet using multiple methods we here consistently find that zonulin plays a negligible role in human BBB permeability.

Zonulin is a member of the MASP (mannose-binding lectin-associated serine protease) family of proteins and elevated serum zonulin levels have been reported in a number of neurological conditions such as multiple sclerosis7 and Alzheimer’s disease.8 The significance of zonulin upregulation in these neurological diseases is not certain. Specifically, it is not clear whether zonulin is an epiphenomenon, or has an effect on the brain, whether directly or mediated through the gut-brain axis.

To study the association between zonulin and BBB permeability in healthy individuals and patients with neurological disease, we employed two techniques to measure permeability across a range of molecular weights: QAlb, or the quotient of cerebrospinal fluid (CSF) to serum albumin (60,000 daltons) in Study A and dynamic contrast enhanced magnetic resonance imaging (DCE-MRI) with gadobutrol tracer (600 daltons) in Study B (Supplemental Methods). Participant characteristics are shown in Table 1.

Out of a total of 217 cases (including people with neurological conditions and control individuals) across both studies, 58 (27%) individuals tested negative for serum zonulin using a novel enzyme-linked immunosorbent assay (ELISA) (Supplemental Methods). Serum zonulin concentration followed a non-Gaussian distribution with a range of 0- 11µg/mL. There were no effects of sex, age or disease status (disease versus control) on zonulin concentrations (ANCOVA, F(3,216) = 1.06, p=0.37). In order to assess zonulin using a complementary dual approach, we determined the haptoglobin phenotype (Supplemental Methods) across all participants from both Studies A and B. Since zonulin is a precursor of haptoglobin-2, one would expect an increase in serum zonulin concentration with *HP2* allele dosage (*HP1-1* < *HP2-1* < *HP2-2*), and this pattern was indeed observed (Supplemental Figure 1), with a significant difference in zonulin between haptoglobin phenotypes (ANOVA, F(2,216)=43.8, p<0.0001). However it is important to note that nine out of 34 *HP1-1* cases tested positive for zonulin (Supplemental Figure 1), indicating that the ELISA was cross-reacting against other ZFP (zonulin family of proteins) members, as established previously with other zonulin ELISAs.9 This highlights the importance of adopting a dual approach to assessing zonulin by using both ELISA and haptoglobin phenotyping.

In Study 1, QAlb was significantly higher with age (p=0.0002), higher in males *versus* females (p<0.0001) and higher in people with neurological disease *versus* healthy controls in univariable analysis (p=0.0003, Figure 1A-C). A multivariable linear regression, controlling for age, sex and disease status, showed that serum zonulin did not associate with QAlb (p=0.92, Table 2 and Figure 1D). Regressing QAlb on the presence or absence of the *HP2* allele (*HP2-2* and *HP2-1* individuals versus *HP1-1* individuals) instead of zonulin concentration, using the same covariates, also showed no association (p=0.313, data not shown).

In Study 2, Ki was significantly higher with age (p=0.007), was not different between males *versus* females (p=0.23) and was significantly higher in people with multiple sclerosis *versus* healthy individuals in univariable analysis (p=0.001, Figure 1E-G). A multivariable linear regression controlling for sex, disease status and age, showed that serum zonulin was not positively associated with Ki (Table 2 and Figure 1H). Regressing Ki on the presence or absence of the *HP2* allele (*HP2-2* and *HP2-1* individuals versus *HP1-1* individuals) instead of zonulin concentration also showed no association (p=0.50, data not shown). Most circulating mediators relevant to pathology, such as cytokines, lipopolysaccharide, viral nucleic acids, complement components, kinins, prostaglandins, hormones and neuro-active monoamines2 have molecular weights below 60kDa. It is currently not technically possible to measure permeability of the human BBB to larger molecular weight substances *in vivo*,2 yet this is important for immunoglobulin G which has a molecular weight of 150kDa. Hence, we examined BBB permeability to larger molecules (70kDa and 150kDa) using a well-established human brain endothelial cell line (hCMEC/D3) BBB model (Supplemental Methods).

The effect of zonulin on the permeability of the hCMEC/D3 monolayer to 70kDa and 150kDa fluorescent dextrans was assessed at 1h intervals up to 6h after treatment with recombinant zonulin. A 1:1 mixture of the cytokines TNF-α (tumour necrosis factor-alpha) and IFN-γ (interferon-gamma) was used as a positive control. Compared to vehicle control wells, TNF-α and IFN-γ significantly increased the permeability of the hCMEC/D3 monolayer to the 70kDa (Figure 2A,C) and 150kDa dextrans (Figure 2B,D). Monolayers treated with zonulin showed no difference in permeability to the 70kDa (Figure 2A,C) and 150kDa dextrans (Figure 2B,D) compared with controls.

This is the first study to examine the role of zonulin in BBB permeability in humans. A major strength of this work, important in confirming the absence of a significant contribution of zonulin to BBB regulation, is the robustness of findings using different methodologies. Still, it remains possible that local and/or transient changes in concentrations of zonulin at brain capillary surfaces are not well represented by either circulating zonulin levels or haptoglobin phenotype. The simplistic *in vitro* model of the BBB used does not fully recapitulate the anatomy and physiology of the living BBB, and future studies should aim to replicate results using three-dimensional all-human multicellular BBB models and more sophisticated methods for assessing BBB permeability.

Although preclinical studies suggested that zonulin has potential to regulate BBB permeability,3-6 we find no evidence for a significant contribution of zonulin in humans, using a number of technical approaches to account for zonulin and to quantify BBB permeability. This is an important negative finding and suggests that the association of serum zonulin levels with clinical manifestations in various neurological diseases7, 8 is unlikely to be mediated by a direct effect of zonulin on BBB permeability. Other indirect mechanistic pathways such as gastrointestinal permeability linked with the gut-brain axis are more likely to be responsible, as exemplified by zonulin transgenic mice which display neurological abnormalities improved by antibiotic depletion of gut microbiota.10 Future studies should further investigate these pathways and the relationship between zonulin and severity of neurological disease.

**List of abbreviations:**

ANCOVA analysis of covariance

ANOVA analysis of variance

BBB blood-brain barrier

CSF cerebrospinal fluid

DCE-MRI dynamic-contrast enhanced magnetic resonance imaging

ELISA enzyme-linked immunosorbent assay

FITC fluorescein isothiocyanate

HP haptoglobin (gene)

IFN-γ interferon-gamma

kDa kilodalton

Pc permeability coefficient

PS permeability-surface area product

Qalb quotient of cerebrospinal fluid to serum albumin

TNF-α tumour necrosis factor-alpha

ZFP zonulin family of proteins

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**Conflicts of interest:**

None of the authors have any potential competing interests.

**Author Contributions:**

CMS (Southampton, UK) performed or coordinated experiments, performed statistical analysis, and wrote the first version of the manuscript. AV collected Study B’s DCE-MRI data and serum samples in Southampton, UK, and was assisted remotely by HBWL and SC (Copenhagen, Denmark) with the design of the MR acquisition protocol and subsequent analysis. MEW and AVK analysed Study A and B serum samples in Linköping, Sweden, with the zonulin ELISA developed by Bio-Rad in Montpellier, France (PG). ZM and GJP (Portsmouth, UK) helped with the *in vitro* blood-brain barrier model experiments conducted in Southampton, for which AF (Boston, USA) provided recombinant zonulin. IG conceived the study and supervised the work. All authors contributed intellectually and revised the manuscript.

**Ethical approval:**

This work was done after National Research Ethics Service approval (11/SC/0204 and 12/SC/0176) and institutional research ethics approval (ERGO 41084.A1 and 5562).

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**Table 1. Participant characteristics.**

Age is given as the mean, zonulin, QAlb and Ki are given as medians (inter-quartile range).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **Study A** | | **Study B** | |
| **Controls**  **(n=40)** | **Neurological disease†**  **(n= 154)** | **Controls**  **(n=12)** | **Relapsing-remitting multiple sclerosis**  **(n=11)** |
| **Age (years)** | 50.5 | 53.4 | 31.3 | 43.4 |
| **Sex (% female)** | 57% | 47% | 67% | 73% |
| **Haptoglobin phenotype (count)** | | | | |
| *HP1-1* | 7 (17.5%) | 25 (16.2%) | 1 (8.3%) | 1 (9%) |
| *HP2-1* | 18 (45.0%) | 63 (40.9%) | 7 (58.3%) | 5 (45.5%) |
| *HP2-2* | 15 (37.5%) | 66 (42.9%) | 4 (33.3%) | 5 (45.5%) |
| **Zonulin, ng/mL** | 63.0 (290.5) | 58.5 (216.9) | 0.0 (314.4) | 67.5 (323.8) |
| **QAlb** | 0.005 (0.003) | 0.007 (0.01) | - | - |
| **Ki** | - | - | -0.006 (0.03) | 0.06 (0.05) |

**†** Diagnoses for participants with neurological disease in Study A included: inflammatory disease (n=79), degenerative disease (n=13), ischaemic disease (n=13), normal pressure hydrocephalus (n=9), infectious (n=5), headache syndrome (n=5), tumour (n=2), structural (n=2), epilepsy (n=1), idiopathic (n=1), hereditary neuropathy (n=1), metabolic (n=1), vascular (n=1) and unknown (n=21).

**Table 2. Multivariable linear regression results.**

For Study A, using QAlb as a marker of BBB permeability: model fit: F(4,193)=11.6, p<0.0001, R2=0.20, adjusted R2=0.18. For Study B using Ki as a marker of BBB permeability: model fit: F(4,22)=7.99, p=0.001, R2=0.64, adjusted R2=0.56.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **STUDY A: QAlb as a marker of BBB permeability** | | | | | | | | | |
|  | **Unstandardized Coefficients** | | | **Standardized Coefficients** |  | | **95% Confidence Interval for B** | | |
| **Independent variable** | **B** | | **Std. Error** | **Beta** | **t** | **Sig.** | **Lower Bound** | | **Upper Bound** |
| **(Constant)** | -2.354 | | 0.076 |  | -30.976 | 0.000 | -2.504 | | -2.204 |
| **log10-zonulin, ng/mL** | -0.002 | | 0.016 | -0.007 | -0.106 | 0.916 | -0.033 | | 0.03 |
| **Sex** | -0.144 | | 0.035 | -0.279 | -4.086 | **0.000** | -0.214 | | -0.075 |
| **Age** | 0.003 | | 0.001 | 0.168 | 2.475 | **0.014** | 0.001 | | 0.005 |
| **Disease status†** | 0.142 | | 0.042 | 0.221 | 3.379 | **0.001** | 0.059 | | 0.224 |
| **STUDY B: using Ki as a marker of BBB permeability** | | | | | | | | | |
|  | **Unstandardized Coefficients** | | | **Standardized Coefficients** |  | | **95% Confidence**  **Interval for B** | | |
| **Independent variable** | **B** | **Std. Error** | | **Beta** | **t** | **Sig.** | **Lower Bound** | **Upper Bound** | |
| **(Constant)** | -0.002 | 0.011 | |  | -0.185 | 0.855 | -0.024 | 0.02 | |
| **log10-zonulin, ng/mL** | -0.005 | 0.002 | | -0.305 | -2.106 | **0.05** | -0.01 | 0.00 | |
| **Sex** | -0.013 | 0.006 | | -0.312 | -2.175 | **0.043** | -0.026 | 0.00 | |
| **Age** | 0.001 | 0.00 | | 0.321 | 1.92 | 0.071 | 0.00 | 0.001 | |
| **Disease status#** | 0.021 | 0.006 | | 0.529 | 3.202 | **0.005** | 0.007 | 0.034 | |

**†** healthy versus neurological disease

**#** healthy versus multiple sclerosis

**FIGURES**

**Figure 1.** A-D: Study A employed QAlb as a human BBB permeability marker. Qalb was higher with age (A) and in males (B) and individuals with neurological disease (C), in univariable analyses. (D) Multivariable linear regression showed no relationship between zonulin and QAlb. E-H: Study B used DCE-MRI to derive Ki as a measure of human BBB permeability. Ki was significantly higher with age (E), was not different between males and females (F) and was significantly higher in individuals with multiple sclerosis versus healthy individuals (G) in univariable analyses. (H) Multivariable linear regression showed no relationship between zonulin and Ki. Since Ki in healthy individuals is close to zero, negative values may arise due to random noise. No positivity constraint was applied to the data. In A, D, E and H dashed lines represent 95% confidence intervals.

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**Figure 2.** Permeability of human cerebral microvascular endothelial cell (hCMEC/D3) monolayers to 70kDa (A) and 150kDa (B) dextrans in the presence of vehicle (black), TNF-α + IFN-γ as positive control (blue) or zonulin (pink) at hourly intervals over a 6h period. Two-way repeated measures ANOVA revealed that there was a significant main effect of TNF-α + IFN-γ on the Pc for the 70kDa dextran (F(1,3) = 268.4, p < 0.001, ηp2 = 0.989) and 150kDa dextran (F(1,3) = 196.6, p < 0.001, ηp2 = 0.985) but there was no effect of zonulin on the Pc for either 70kDa (F(1,3) = 0.34, p = 0.601, ηp2 = 0.102) or 150kDa dextran (F(1,3) = 0.152, p = 0.723, ηp2 = 0.048). (C-D) The estimated marginal mean (EMM) of the Pc (controlling for time in the two-way repeated measures ANOVA) was higher after cytokine treatment, but similar between zonulin and vehicle-treated wells. NS = not significant, \*\* p < 0.001. All experiments were repeated four times (n=4), each with triplicate wells per condition.

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