Widely used commercial ELISA for human Zonulin reacts with Complement C3 rather than preHaptoglobin2

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Nonstandard abbreviations: HP, haptoglobin; T2D, type 2 diabetes; WHR, waist-to-hip ratio.
Abstract

BACKGROUND. There is increasing evidence for the role of impaired intestinal permeability in obesity and associated metabolic diseases. Zonulin is an established serum marker for intestinal permeability and supposedly identical to pre-haptoglobin2. Here, we aimed to investigate the relationship between intestinal permeability represented by circulating zonulin and metabolic traits related to obesity.

METHODS. Serum zonulin was measured by using a widely used commercially ELISA kit in 376 subjects from the metabolically well-characterized cohort of Sorbs from Germany. In addition, haptoglobin genotype was determined in DNA samples from all study subjects.

RESULTS. The genotype frequencies for haptoglobin genotypes HP1/1, HP1/2 and HP2/2 were 15.8%, 47.6% and 36.6% respectively. Since zonulin concentrations did not correspond to the haptoglobin genotypes we investigated the specificity of the zonulin ELISA assay using immunoprecipitation experiments, mass spectrometry and Western blot analysis. Thus we demonstrated by mass spectrometry that the kit is mainly capturing complement factor C3 and derived fragments. Circulating complement factor C3 was significantly increased in patients with diabetes and obesity and correlated strongly with markers of the lipid and glucose metabolism.

CONCLUSIONS. Our study supports the role of the complement C3 in the pathophysiology of obesity and necessitates critical revision of published data on zonulin as a marker for altered intestinal permeability from studies that have used the ELISA kit investigated here.
Introduction

The "intestinal barrier" is an established term, defined as a functional entity separating the gut lumen from the inner host, and consisting of mechanical, humoral, immunological, muscular and neurological elements. Intestinal barrier dysfunction is a characteristic feature of pathological states such as inflammatory bowel disease, celiac disease, nonalcoholic steatohepatitis and ulcerative colitis [1, 2]. There is also emerging evidence for the role of impaired intestinal permeability in metabolic diseases including obesity and type 2 diabetes (T2D) [3–5]. It has been hypothesized that gut bacteria and bacterial endotoxins may disrupt the intestinal barrier resulting in the so called "leaky gut" [4, 6]. The leakage of toxins, bacterial components or even live bacteria and their transfer to target organs such as adipose tissue might contribute to the development of obesity and T2D [6, 7]. Indeed, numerous studies in mouse models demonstrated that changes in the gut microbiota can alter the gut permeability and lead to an endotoxemia-induced inflammation in adipose tissue, and ultimately to obesity [3, 8, 9]. Results from experimental mouse models are supported by studies in humans by showing an increase in circulating endotoxin levels and circulating bacterial DNA in obese/diabetic patients, likely due to an increased intestinal permeability in affected subjects [10, 11].

Intestinal barrier transport is mainly regulated by structures of the paracellular pathway called tight junctions which form barriers between epithelial cells and regulate the transport of ions and small molecules across the intestinal lumen. Intestinal permeability is a functional feature of the intestinal barrier. It can be measured by analyzing flux rates of inert molecules across the intestinal wall as a whole or across wall components [1]. The gold standard for assessment of intestinal permeability in vivo is an assay combining indigestible large and small oligosaccharides, such as lactulose and mannitol; the larger oligosaccharide,
lactulose, is only transported via the paracellular pathway, whereas the smaller oligosaccharide, mannitol, is taken up freely over the intestinal barrier via the transcellular route. However, these oligosaccharide assays are extensive, laborious and time consuming. Therefore, identifying appropriate biomarkers for intestinal permeability is highly desirable. Fasano et al. identified zonulin as a new tight junction regulating protein which is, functionally, the human counterpart of the Vibrio cholera endotoxin zonula occludens toxin [12, 13]. Later, the same group described zonulin as the precursor of haptoglobin 2 (pre-HP2) [14]. Haptoglobin is a well-known protein involved in the degradation of hemoglobin, whereas the function of its precursor is largely unknown. Haptoglobin is first synthesized into a single chain precursor protein, which is cleaved into a light N-terminal α-chain and heavy C-terminal β-chain. An exon duplication of exons 3 and 4 of the haptoglobin gene differentiates the HP1 from the HP2 allele. Due to this exon duplication the HP2 α-chain is 1.7kb longer than in the HP1 allele. Haptoglobin is active as tetramer consisting of 2 α- and 2 β-chains linked by disulfide bonds, resulting in three possible genotypes: homozygous HP1/1 and HP2/2 as well as heterozygous HP1/2 [15, 16]. About 15 % of the Caucasian population are homozygous for HP [16, 17]. Zonulin as pre-HP2 reversibly opens tight junctions and is upregulated in diseases such as celiac disease and type 1 diabetes (T1D) [14, 18]. Serum zonulin concentrations are also increased in T2D and obesity [19–21] and strong correlations were observed with various metabolic markers, including fasting plasma glucose, IL-6, HDL, and triglyceride (TG) levels [19–21]. However, the rather small sample sizes in these studies warrant further analyses to validate and strengthen the initial findings.

Here, we aimed to characterize the relationship between circulating serum zonulin and traits related to obesity in a metabolically well-characterized cohort of Sorbs from Germany. To measure zonulin, we used the widely acknowledged commercially
available ELISA kit (Immundiagnostik, Bensheim, Germany). In addition, we
determined the haptoglobin genotypes in the entire cohort. Due to a lack of
correspondence between the observed circulating zonulin concentrations and the
haptoglobin genotypes in our study cohort, we further investigated the identity of the
product captured by the commercial ELISA assay. We revealed that the ELISA kits
used in the present study actually targeted the complement factor C3 and C3-derived
fragments, which were strongly associated with altered lipid and glucose metabolism.

Materials and Methods

Study subjects

All subjects are part of a sample from an extensively clinically characterized
population from Eastern Germany, the Sorbs [22–24][1–3]. Extensive phenotyping
included standardized questionnaires to assess past medical history and family
history, collection of anthropometric data (weight, height, waist-to-hip ratio (WHR)),
and an oral glucose tolerance test. Glucose was assessed by the Hexokinase
method (Automated Analyzer Modular, Roche Diagnostics, Mannheim, Germany)
and serum insulin was measured using the AutoDELFIA Insulin assay (PerkinElmer
Life and Analytical Sciences, Turku, Finland). Total serum cholesterol and TG
concentrations were measured by standard enzymatic methods (CHOD-PAP and
GPO-PAP; Roche Diagnostics). Serum LDL-C and HDL-C concentrations were
determined using commercial homogeneous direct measurement methods (Roche
Diagnostics). All assays were performed in an automated clinical chemistry analyzer
(Hitachi/ Roche Diagnostics) at the Institute of Laboratory Medicine, University
Hospital Leipzig.

All blood samples were taken in the morning after an overnight fast and stored at -
80°C until analyses. From the 1040 Sorbs enrolled in the cohort, a subgroup of 376
subjects was genotyped for haptoglobin and provided blood samples for zonulin measurements (Table 1). Main metabolic characteristics of the study subjects are summarized in Table 2.

**Table 1: Main characteristics of the study cohort included in zonulin measurements.**

<table>
<thead>
<tr>
<th></th>
<th>all</th>
<th>female</th>
<th>male</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n</strong></td>
<td>376</td>
<td>233</td>
<td>143</td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td>53.3±16.2</td>
<td>52.7±15.8</td>
<td>54.2±16.8</td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>28.1±5.6</td>
<td>27.9±5.9</td>
<td>28.5±5.1</td>
</tr>
<tr>
<td><strong>NGT</strong></td>
<td>191 (50.8%)</td>
<td>124 (53.2%)</td>
<td>67 (46.8%)</td>
</tr>
<tr>
<td><strong>IGT</strong></td>
<td>79 (21.0%)</td>
<td>49 (21.0%)</td>
<td>30 (21.0%)</td>
</tr>
<tr>
<td><strong>T2D</strong></td>
<td>106 (28.2%)</td>
<td>60 (25.8%)</td>
<td>46 (32.2%)</td>
</tr>
</tbody>
</table>

Age and BMI are mean ± SD, NGT, IGT, T2D as absolute number (percentage); NGT: normal glucose tolerance; IGT: impaired glucose tolerance; IFG: impaired fasting glucose.

**Table 2: Main characteristics of the study participants.**

<table>
<thead>
<tr>
<th></th>
<th>NGT</th>
<th>IGT</th>
<th>T2D</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n</strong></td>
<td>191</td>
<td>79</td>
<td>106</td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td>45.4±15.6</td>
<td>59.7±29.4**</td>
<td>62.9±11.2**</td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>25.8±5</td>
<td>29.7±4.6**</td>
<td>30.97±5.53**</td>
</tr>
<tr>
<td><strong>WHR</strong></td>
<td>0.84±0.10</td>
<td>0.92±0.09</td>
<td>0.94±0.09**</td>
</tr>
<tr>
<td><strong>FPG (mmol/l)</strong></td>
<td>5.15±0.50</td>
<td>5.68±0.56**</td>
<td>7.72±2.43**</td>
</tr>
<tr>
<td><strong>120 min PG (mmol/l)</strong></td>
<td>5.00±1.40</td>
<td>8.95±0.98**</td>
<td>5.78±6.86○ ○</td>
</tr>
<tr>
<td><strong>Triglycerides (mmol/l)</strong></td>
<td>1.29±0.94</td>
<td>1.56±0.92*</td>
<td>1.87±1.24**</td>
</tr>
<tr>
<td><strong>Fasting Insulin (pmol/l)</strong></td>
<td>35.90±25.98</td>
<td>53.56±25.98**</td>
<td>62.38±43.07**</td>
</tr>
<tr>
<td><strong>120 min Insulin (pmol/l)</strong></td>
<td>148.39±133.58</td>
<td>409.28±232.81**</td>
<td>182.22±272.34○ ○</td>
</tr>
<tr>
<td><strong>HDL (mmol/l)</strong></td>
<td>1.69±0.40</td>
<td>1.56±0.36*</td>
<td>1.43±0.39** ○</td>
</tr>
<tr>
<td><strong>LDL (mmol/l)</strong></td>
<td>3.20±0.96</td>
<td>3.70±1.06**</td>
<td>3.33±0.95 ○</td>
</tr>
<tr>
<td><strong>Total cholesterol (mmol/l)</strong></td>
<td>5.12±1.03</td>
<td>5.57±1.18*</td>
<td>5.27±0.99</td>
</tr>
<tr>
<td><strong>HOMA IR</strong> (pmol/l*mmol/l)</td>
<td>1.34±1.06</td>
<td>2.30±1.1**</td>
<td>3.58±2.87** ○ ○</td>
</tr>
<tr>
<td><strong>HOMA IS (ratio)</strong></td>
<td>1.07±0.76</td>
<td>0.67±0.78**</td>
<td>0.68±1.63**</td>
</tr>
<tr>
<td><strong>CRP (mg/l)</strong></td>
<td>2.22±4.20</td>
<td>3.19±4.54</td>
<td>3.87±4.76**</td>
</tr>
<tr>
<td><strong>Zonulin (ng/ml)</strong></td>
<td>67.25±25.45</td>
<td>71.88±29.36</td>
<td>81.78±25.31** ○ ○</td>
</tr>
</tbody>
</table>

*: p<0.05 vs NGT; **: p<0.01 vs NGT
○: p<0.05 vs IGT; ○○: p<0.01 vs IGT

All data given as mean ± standard deviation, NGT: normal glucose tolerance, IGT: impaired glucose tolerance, T2D: type 2 diabetes, CRP: C-reactive protein, HDL: High density lipoprotein, LDL: Low density lipoprotein, FPG: Fasting plasma glucose, WHR: Waist to hip ratio.
The study was approved by the ethics committee of the University of Leipzig and all subjects gave written informed consent before taking part in the study.

**ELISA measurements**

Circulating zonulin was measured by a competitive ELISA (Immundiagnostik AG, Bensheim, Germany) in serum samples of 376 individuals.

**Genotyping**

Haptoglobin genotypes were determined by PCR using a method adapted from Koch et al. [17]. Briefly, two primer pairs were used: A (5’-GAGGGGAGCTTGCTTTCCATTG-3’) and B (5’-GAGATTTTTGAGCCCTGGCTGGT-3’) as well as C (5’-CCTGCCTCGTATTAACTGCACC AT-3’) and D (5’-CCGAGTGCTCCACATAGCCATGT-3’). The primer pair A/B generates two bands: a 1,757-bp allele 1 specific band and a 3,481-bp allele 2 specific band. The primer pair C/D produces one allele 2 specific band of 349 bp. The combination of the bands allows a reproducible typing of the two common haptoglobin genotypes HP1 and HP2. In contrast, no band is detectable for the rare HP deletion genotype, present in ~0.1% Caucasians [16].

**Characterization of ELISA products**

**Immunoprecipitation**

Immunoprecipitation (IP) of target protein from serum samples utilizing the immobilized anti-zonulin antibodies on the ELISA plates was performed. Undiluted serum samples with highest and lowest concentrations of zonulin, as measured using
the same ELISA kit, were transferred to separate wells and incubated and washed
according to the manufacturer’s protocol. Afterwards, 50 µl of hot (95°C) SDS sample
buffer with β-mercaptoethanol were added to the wells and incubated for 5 minutes to
release bound proteins from the immobilized capturing antibody. To increase the final
protein concentration, the initial 50 µl of SDS sample buffer were successively used
in further wells containing either high or low zonulin serum samples, respectively.
Proteins from 20 µl of each pooled sample (high or low zonulin) were separated by
SDS-PAGE using precast Bolt 4-12% Bis-Tris Plus gels (ThermoFisher, Waltham,
MA, USA). Proteins were stained using the Pierce Silver Stain for mass spectrometry
(ThermoFisher).

**Mass spectrometry**

To identify proteins isolated from serum samples using the zonulin ELISA kit as
described above, bands were excised from silver-stained gels and in-gel digested
with trypsin following a standard protocol [25]. After enzymatic digestion, the peptide
mixtures were immediately analyzed by LC/MS/MS on an U3000 RSLC nano-HPLC
system (Thermo Fisher Scientific) coupled to an Orbitrap Fusion Tribrid mass
spectrometer (Thermo Fisher Scientific). Samples were loaded onto a pre-column
(RP-C8, 300 µm * 5 mm, 5 µm, 100 Å, Thermo Fisher Scientific) and washed with
water containing 0.1% (v/v) TFA for 15 min, before the peptides were separated on
the separation column (RP-C18, 75 µm * 250 mm, 2 µm, 100 Å, Thermo Fisher
Scientific) using gradients from 1% to 40% (v/v) B (45 min), 40% to 85% (v/v) B (5
min) followed by 85% B (5 min), with solvent A: 0.1% (v/v) formic acid (FA) in water
and solvent B: 0.08% (v/v) FA in acetonitrile. Data were acquired using data-
dependent MS/MS mode where each high-resolution full-scan in the orbitrap (m/z
198 to 1,500; R = 120,000) was followed by high-resolution product ion scans in the
orbisrap (higher energy collision-induced dissociation (HCD), 27% normalized
collision energy, R = 15,000, isolation window 2 Th) within 5 s, starting with the most
intense signal in the full-scan mass spectrum.

Data analysis was performed using the Proteome Discoverer 2.1 (Thermo Fisher
Scientific). MS/MS data of precursor ions (m/z range 350-5,000) were searched
against the Swissprot Database (version 11/2016, taxonomy human, 20,082 entries)
and a contaminant database using Sequest HT. Mass accuracy was set to 5 ppm
and 20 mmu for precursor and fragment ions, respectively. Carbamidomethylation of
cysteines was set as fixed modification; oxidation of methionines and N-terminal
acetylation were set as variable modifications, two missed cleavages of trypsin were
allowed. Results were filtered for non-contaminant proteins identified by at least three
unique highly confident peptides (peptide FDR ≤ 1%).

Western blot analysis

Western blot experiments were performed to validate the results of mass
spectrometric data analysis. Gels were blotted on a PVDF membrane and Western
blots were probed with anti-C3 β-chain (1:2000) (Biozol, Eching, Germany) and anti-
haptoglobin (1:1000) (Abcam, Cambridge, UK) antibodies. Purified C3c from plasma
(Athens Research, Athens, Georgia, USA) was used as a positive control. Incubation
with primary and secondary antibodies (HRP-conjugated) was done for 90 minutes at
room temperature. Blots were visualized by enhanced chemiluminescence using
Pierce ECL Western Blotting Substrate (ThermoFisher).

Statistical analysis

Statistical analysis was performed using with SPSS 24 (IBM). All non-normally
distributed metric parameters were log transformed to generate a Gaussian normal
distribution. Spearman’s rank correlation method was used to assess the relationship between metabolic traits. To test for significant differences in distribution for ordinal values, the Kruskal-Wallis-test was used. Exact differences between two groups were tested by both the Mann-Whitney-U test and unpaired student’s t-test. In addition, multiple linear regression analyses were done to assess the linear relationship between continuous variables and genotypes. For all tests, a p-value <0.05 was considered to be statistically significant. These statistical tests were used as referenced in the results.

Results

Haptoglobin genotype

Haptoglobin genotype HP1/1 was present in 15.8% of the subjects, HP1/2 in 47.6% and HP2/2 in 36.6%. These frequencies are comparable to the distribution of HP genotypes in Caucasians reported by others [16, 17]. We tested the association of the HP genotypes with various anthropometric and metabolic traits in all study subjects. The analysis essentially revealed that hemoglobin levels significantly increase with the presence of at least one HP2 allele (p=0.004 over all three groups, p=4.2x10^{-4}) between HP1 homozygote and HP2 carriers). Furthermore, we observed that the total protein concentration in the urine significantly differed between the three groups, with an increase in the HP2 carriers having increased concentrations (p=0.027). Interestingly, mean triiodothyronine (rT3) levels were lower in the HP1/1 group than in the HP2/2 group (p=0.012) and in accordance, an increase in administered thyroid gland hormones (p=0.023) was observed.
Zonulin ELISA data do not reflect HP genotype distribution

Strikingly, there were no significant differences in levels of the zonulin ELISA signal between the three haptoglobin genotype groups (Figure 1; p=0.153 using ANOVA, p=0.07 for the t-test comparing log transformed zonulin signals between HP1/1 vs. HP1/2 + HP2/2). Assuming that the protein measured by the kit is zonulin (i.e. pre-HP2), subjects with the HP1/1 genotype, which has a frequency of ~15% in Caucasians [16, 17], were expected to have no detectable zonulin levels. As the zonulin concentrations measured in patient sera using the zonulin ELISA kit clearly did not reflect the HP genotype distribution we therefore concluded that the protein measured by the kit is not identical to zonulin as pre-HP2. Consequently, we aimed to unravel the identity of the protein(s) detected by the alleged zonulin ELISA kit.

Identification of the (major) protein bound by the zonulin ELISA capturing antibody

To identify proteins bound and therefore quantified by the capturing antibody deployed in the zonulin ELISA kit, which was used in this and many other studies (Supplementary Table 1), we performed an immunoprecipitation experiment using patient sera and the immobilized antibody of the ELISA kit as supplied. After incubation of the immobilized ELISA kit antibodies with selected patient sera representing the highest and lowest measured ELISA signals in the cohort, the captured proteins were separated by SDS-PAGE. Silver staining revealed multiple bands, with the most intense band at ~70 kDa and further prominent bands at ~55 kDa, ~180 kDa and >180 kDa (Figure 2A). This band pattern was incompatible with a band pattern that would be expected for pre-HP2 or haptoglobin-derived proteins and further supported the results demonstrating the lack of correspondence of the captured protein with HP genotypes. To unequivocally identify all major proteins
captured by the ELISA kit, protein bands were cut (Figure 2A) and subjected to MS analysis after tryptic digestion (Supplementary Table 2). Mass spectrometry demonstrated that bands 1 (>180kDa), 2 (~150 kDa), 3 (~70 kDa) were all very likely representing the C3 protein or cleavage products derived from the C3 protein, such as the C3 precursor (187 kDa), C3c (144 kDa) and the C3 β-chain (71 kDa) (Figure 2C). Furthermore, the 55 kDa band was identified as properdin or factor P (MW 51 kDa). To validate results from mass spectrometry, we performed Western blot analyses and indeed, the major band at 70 kDa was clearly detected by the anti-C3 β-chain antibody (Figure 2B). Importantly, the band intensities clearly reflected the signal intensities measured using the zonulin ELISA, with high-level “zonulin” sera yielding very intense C3 β-chain bands after immunoprecipitation, and sera with a low ELISA signal resulting in low Western blot signals.

Circulating complement factor C3 correlates with metabolic traits

In a sample of 376 subjects tested using the purchased zonulin ELISA, the product (circulating C3) was measured in a mean concentration of 72.2 ± 27.2 ng/ml (mean ± standard deviation), ranging from 17.8 to 207.1 ng/ml. The C3 protein was significantly increased in subjects with T2D (81.78 ± 25.31 ng/ml) compared to subjects with normal glucose tolerance (67.25 ± 25.45 ng/ml, Mann-Whitney-U test; p=2.1x10^{-8}) or impaired glucose tolerance (71.88 ± 29.36 ng/ml, p=0.0017) (Figure 3A). Additionally, lean subjects had significantly lower values (65.64 ± 25.23 ng/ml) than subjects with overweight (74.20 ± 30.68 ng/ml, p=0.0082) or obesity (76.24 ± 24.17 ng/ml, p=7.0x10^{-5}) (Figure 3B). We observed no gender differences or any correlations with age (data not shown). Circulating complement factor C3 correlated with traits related to glucose and lipid metabolism (Spearman’s rank correlation test, adjusted for age, sex and BMI; Table 3). It was positively correlated with BMI, HOMA-
IR and -IS and fasting plasma glucose (Table 3). Strong correlations were also observed for lipid metabolism parameters, such as TG levels, total cholesterol, LDL and apolipoprotein B (Table 3). Correlations were tested for a total of 95 accessible traits. After Bonferroni correction for multiple testing (adjusted p-value for significance p < 5.2x10^{-4}), correlations for BMI (p=1.2x10^{-5}), fasting glucose (p=9.0x10^{-6}), TG (p=4.2x10^{-10}), total cholesterol (p=3.3x10^{-5}) and apolipoprotein B (p=2.3x10^{-5}) remained statistically significant.

Table 3: Correlation of complement factor C3 with metabolic phenotypes.

<table>
<thead>
<tr>
<th></th>
<th>Non-adjusted</th>
<th>Adjusted for age, sex, BMI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>p-value</td>
</tr>
<tr>
<td>Anthropometric trait</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>0.31</td>
<td>0.55</td>
</tr>
<tr>
<td>Sex</td>
<td>-0.005</td>
<td>0.93</td>
</tr>
<tr>
<td>BMI</td>
<td>0.221</td>
<td>1.2x10^{-3}</td>
</tr>
<tr>
<td>WHR</td>
<td>0.172</td>
<td>7.3x10^{-4}</td>
</tr>
<tr>
<td>Glucose metabolism</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (0 min)</td>
<td>0.294</td>
<td>6.6x10^{-9}</td>
</tr>
<tr>
<td>Glucose (120 min)</td>
<td>-0.039</td>
<td>0.45</td>
</tr>
<tr>
<td>Insulin (0 min)</td>
<td>0.173</td>
<td>7.7x10^{-4}</td>
</tr>
<tr>
<td>Insulin (120 min)</td>
<td>-0.039</td>
<td>0.46</td>
</tr>
<tr>
<td>HOMA IR</td>
<td>0.244</td>
<td>2.0x10^{-6}</td>
</tr>
<tr>
<td>HOMA IS</td>
<td>-0.243</td>
<td>2.0x10^{-6}</td>
</tr>
<tr>
<td>Lipid metabolism</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglycerides</td>
<td>0.370</td>
<td>6.5x10^{-14}</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>0.219</td>
<td>1.5x10^{-5}</td>
</tr>
<tr>
<td>LDL</td>
<td>0.182</td>
<td>3.4x10^{-4}</td>
</tr>
<tr>
<td>HDL</td>
<td>-0.136</td>
<td>7.7x10^{-3}</td>
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<tr>
<td>Apolipoprotein B</td>
<td>0.247</td>
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</tr>
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<td>Adipokines</td>
<td></td>
<td></td>
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<tr>
<td>Adiponectin</td>
<td>-0.215</td>
<td>2.2x10^{-8}</td>
</tr>
<tr>
<td>Progranulin</td>
<td>0.151</td>
<td>3.0x10^{-3}</td>
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<tr>
<td>Vaspin</td>
<td>0.027</td>
<td>0.6</td>
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<tr>
<td>Chemerin</td>
<td>0.103</td>
<td>4.3x10^{-2}</td>
</tr>
<tr>
<td>FGF21</td>
<td>0.165</td>
<td>2.1x10^{-3}</td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-reactive protein</td>
<td>0.232</td>
<td>4.0x10^{-6}</td>
</tr>
<tr>
<td>Total protein</td>
<td>0.124</td>
<td>1.5x10^{-2}</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>0.201</td>
<td>7.4x10^{-3}</td>
</tr>
<tr>
<td>Uric acid</td>
<td>0.176</td>
<td>5.2x10^{-4}</td>
</tr>
</tbody>
</table>
**bold**: significant correlations after adjustment, **underlined**: significant after Bonferroni adjustment for multiple testing \((p<5.2\times 10^{-4})\); \(r\): Spearman rank correlation coefficient, \(p\): significance level; BMI: Body Mass Index; HDL: High density lipoprotein, LDL: Low density lipoprotein, WHR: Waist to hip ratio, FGF21: Fibroblast growth factor 21, HOMA-IR/IS: Homeostasis Model Assessment Insulin Resistance/ Insulin Sensitivity.

**Discussion**

The role of impaired intestinal permeability in metabolic diseases including obesity and T2D has recently been acknowledged in multiple studies [3, 4, 26]. The tight junction regulator zonulin, which was identified as pre-HP2 by Tripathi et al. [14], is an established circulating marker of intestinal permeability in humans [27, 28]. Here, we aimed to investigate the relationship between intestinal permeability, represented by circulating zonulin, and metabolic traits related to obesity and T2D. We set out to measure zonulin with a widely used commercially available ELISA kit in a metabolically well-characterized cohort of Sorbs from Germany. Considering the identity of zonulin as pre-HP2, we also genotyped the haptoglobin gene in DNA samples from all subjects. The genotype frequency of the HP1/1 genotype corresponded to previously reported frequencies of 15\% in a general Caucasian population [16, 17]. Assuming that the measured zonulin is identical with pre-HP2 as reported previously [13], we expected subjects with the HP1/1 genotype to have no detectable, or if taking into account cross-reactions, at least significantly lower zonulin levels. However, concentrations measured in the HP1/1 homozygous subjects were comparable with those found in HP2/2 and HP1/2 carriers.

The epitope used to generate the capture antibody in the zonulin ELISA is based on the sequence GGVLVQPG published by Wang et al. [29] (communication with customer support; Immundiagnostik AG, Bensheim, Germany), which was initially thought to represent the N-terminal sequence of fetal zonulin [29]. However, this sequence is not present in pre-HP2, which has since been proposed to be zonulin by
the same authors [14]. According to the authors, the discrepancies between the
previously reported zonulin sequence and the pre-HP2 sequence may be due to
intraspecies variability associated with a high zonulin mutation rate or due to
sequencing errors at that time [14]. In summary, the commercially available
competitive ELISA does very likely not detect preHP2 or zonulin, but rather unknown
targets bound by the antibody raised against the sequence reported by Wang et al
[29]. Based on database searches, the epitope would most likely correspond to Glu-
Rich2, a protein which shares 7 out of 8 amino acids. The zonulin/preHP2 binding
compact CD163 shows some conformity with the epitope [30]. Yet, our mass
spectrometry data did not provide any evidence for either protein, but clearly
indicated that the major proteins targeted by the ELISA are the complement factor C3
and C3 derived fragments, in particular the C3 beta chain (Supplementary Table 2
and Figure 2). Properdin, which was also identified in immunoprecipitation
experiments, was likely co-immunoprecipitated, as this serum protein binds the
complex of C3b and activated complement factor B to positively regulate complement
activation via the alternative pathway [31].
Complement factor C3 is the merging point of the three routes of the complement
system pathways and is a well-known protein involved in various aspects of the
primary immune response [32]. By measuring circulating C3 beta chain
concentrations in subjects, whose metabolic phenotypes have been extensively
characterized, our data suggest that it is upregulated both in diabetic and obese
patients. This is in line with previously reported findings showing associations
between C3c levels and insulin resistance as measured by HOMA-IR [33]. Moreover,
C3 is an established player in lipid metabolism [34, 35]. It is not only produced by
hepatocytes, but also by macrophages and adipocytes and it binds to various
lipoprotein particles including Apo-A1 complexes, chylomicrons and HDL and is
potentially involved in the transport of this molecules [34]. Furthermore, C3a-desArg, a constitutively produced C3 fragment (Figure 2C) more commonly known as Acylation-stimulating protein (ASP), is a lipogenic hormone involved in lipid storage and energy homeostasis [36]. ASP is increased in lean and obese diabetic patients [37]. Knockout of the C3a-desArg receptor CC3aR-/- prevents mice from diet-induced insulin resistance [38]. In addition, treatment of these knockout mice as well as knockout rats with C3aR (and C51R) peptide antagonists results in elevated plasma triglycerides [34, 38]. Our data further support a role for C3 in lipid metabolism by showing in various linear stepwise regression models that triglyceride levels and fasting glucose are the strongest independent available variables explaining the observed variance in C3 (Supplementary Table 3). It is noteworthy that numerous studies employing the ELISA kit used in this study reported associations of circulating zonulin with obesity and diabetes similar to our results [19–21], thus strengthening the metabolic correlations observed in our study. Nonetheless, we strongly believe that many of these studies using zonulin as a marker for intestinal permeability would need to revise, or at least reconsider their conclusions (please see Supplementary Table 1 which shows, to the best of our knowledge, a complete listing of all PubMed listed publications using the respective ELISA kit). Rather than showing any relation to intestinal permeability, studies employing the here tested zonulin ELISA kit most likely suggest associations between complement C3 and metabolic traits, although it cannot be ruled out that C3, as a major complement system factor, might be associated with increased intestinal permeability. The factor C3 strongly correlates with insulin resistance [1] and elevated C3 may also be a response to inflammation and bacteria from the “leaky gut” [7, 39, 40]. Further studies are warranted to establish a mechanistic link between C3, intestinal permeability and insulin resistance. Finally, it is also of note, that e.g. in Germany, the zonulin kits analyzed in
our lab are approved for in-vitro diagnostics of altered intestinal permeability (http://www.immundiagnostik.com/fileadmin/pdf/Zonulin_K5601.pdf). It is questionable, that even after publication of the protein identity of zonulin as pre-HP2, the commercial ELISA is still sold as specific for zonulin without mention of the inconsistencies in sequence of pre-HP2 and the peptide used for immunization.

In conclusion, our study supports the role of the complement C3 $\beta$-chain in the pathophysiology of obesity and suggests reconsidering commercially available ELISA kits when investigating zonulin as a marker for altered intestinal permeability.

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Author contributions

LS, PK and JTH conceived the study, designed and conducted experiments, analyzed data and wrote the paper. AT recruited patients. CHI performed mass spectrometry experiments. AC, HH, DS and MS interpreted and analyzed data. All authors discussed results, edited and commented on the manuscript. All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

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Supporting Information

S1 Table: Publications using the analyzed ELISA kit

S2 Table: Mass spectrometry results of tryptic digests

S3 Table: Multiple stepwise linear regression analysis
Fig 1. Distribution of zonulin ELISA values according to haptoglobin genotypes.

Data is presented as boxplots with Turkey-Whiskers and outliers.
Fig 2. A) Silver stain of immunoprecipitated ELISA products and B) Western blot analyses using an anti-C3 β-chain antibody. (C) Proteolytic processing and resulting fragments of complement C3 protein with corresponding molecular weights. Commercially available C3c protein isolated from human plasma was run as positive control, high: pooled IP samples of sera that gave highest ELISA signals; low: pooled IP samples of sera that gave lowest ELISA signals; neg: negative control using dilution buffer from the ELISA kit; tracer: competitive tracer reagent from the ELISA kit. Letters in (A) indicated bands analyzed by MS after tryptic in gel digestion (Supplementary Table 2).
Fig 3. A) Mean zonulin ELISA values according to glucose tolerance groups B) mean ELISA values according to BMI groups. **: p<0.01; ***: p<0.001
NGT: normal glucose tolerance, IGT: impaired glucose tolerance, T2D: type 2 diabetes